MODULATION OF STRESS INDUCED BIOCHEMICAL ALTERATIONS THROUGH DIETARY CONSTITUENTS

THESIS

SUBMITTED FOR THE AWARD OF THE DEGREE OF

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IN

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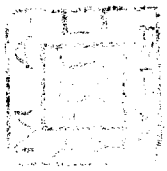

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Certificate

This is to certify that the work presented in this thesis entitled **"Modulation of Stress Induced Biochemical Alterations Through Dietary Constituents"** is an original work done by **Mr. S. M. Kashif Rais Zaidi** under my supervision and is suitable for submission for the award of Ph.D. Degree in Biochemistry.

(Naheed Banu, Ph.D.)
Associate Professor

In loving memory
of
my brother who is
no more to see this
achievement

Dedicated

to

My Parents

Acknowledgement

In the name of Allah the most beneficent and most merciful

In the process of discovering, working and studying a few remarkable aspects of science, I came across a whole new plethora of emotions, a gamut of ideas and whole a new concept of human relations. The concept that my PhD work is also a period of great understanding and remarkable support from people around me.

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CONTENTS

INTRODUCTION

1. INTRODUCTION

Today there is renewed interest in the effects of food upon health, both beneficial and deleterious, and in the potential that certain constituents of food may have upon the prevention of chronic diseases, such as heart disease, cancer, diabetes and osteoporosis. The food supply has come under increased scrutiny for its palatability, safety and efficacy. There is an explosion of research on phytochemicals, and how they may work. Foods are now viewed not only as source of calories, vitamins and minerals, but as having potential effects well beyond.

Immobilization / restraint stress is an easy and convenient method to induce both psychological (escape reaction) and physical stress (muscle work) resulting in restricted mobility and aggression. Recently various stresses have been associated with enhanced free radical generation causing oxidative stress. One of the most important consequences of the generation of free radicals is the peroxidation of membrane lipids. Enhanced free radical production and lipid peroxidation in terms of MDA have been well documented in stress. This is probably induced due to decreased activities of free radical metabolizing enzymes such as SOD, GST and catalase. Moreover, stress has been suggested to decrease the level of glutathione (GSH) and vitamin C, which plays an important role in protection of tissues from oxidative damage. Next to the huge group of polyphenols, especially tocopherols, ascorbic acid and carotenoids have been associated with antioxidative properties.

To protect molecules against toxic free radicals and other ROS, cells have developed antioxidant defenses that include the enzymes superoxide dismutase (SOD), which dismutates superoxide; catalase and glutathione peroxidase, which destroy toxic peroxides, and small molecules including glutathione. External sources of antioxidant nutrients that are essential for antioxidant protection include vitamins C and E, vitamin A / provtamin A and the mineral selenium, a component of selenium dependent glutathione peroxidase.

In the present study the oxidative stress generated by the restraint stress was measured in terms of free radical scavenging enzyme activities like superoxide dismutase, catalase, glutathione-S-transferase and thiobarbituric acid reactive substances (TBARS).

The antioxidant potential both the prophylactic and curative of vitamin A, E and C individually and in combination (vitamin E+C) was evaluated in terms of activities of SOD, GST, CAT and the levels of uric acid, glucose and MDA measurements on restraint induced oxidative stress. Various other biochemical markers like GOT, GPT, and alkaline phosphatase were also determined both in tissues and plasma samples. Further, the role of various indigenously used dietary components like garlic (*Allium sativum*), onion (*Allium cepa*) was also evaluated. The aqueous extract of *S. nigrum* leaves was also studied as it is used for various therapeutic purposes in India. The antioxidant role of *S. nigrum* has not been worked out in details, thus it was included in the present study and its prophylactic and curative role was evaluated in oxidative stress through measurement of oxidative status of the rats both before and after immobilization stress.

The results of the study are likely to contribute to understanding the potential of antioxidant vitamins, extract of garlic, onion and *S. nigrum* in preventing / alleviating stress induced diseases involving oxidative damage to cellular constituents.

REVIEW OF LITERATURE

2. REVIEW OF LITERATIURE

2.1 Antioxidant nutrients

Free radicals are generated continuously in the body due to both normal metabolism and diseases (Gate *et al*, 1999). When an imbalance occurs between oxidants and antioxidants in favor of the oxidants, excess reactive oxygen species are formed; these may contribute to the aging process as well as to chronic diseases such as cancer and coronary heart disease (Benzie *et al.*, 2000). Numerous epidemiological studies have indicated that diets high in fruits and vegetables play a role in reducing the risk of several chronic diseases (Liu *et al.*, 2000; Zeigler *et al*, 1991). It is possible that antioxidant nutrients in the fruits and vegetables can prevent certain damages from harmful free radicals that are produced in the body. However, it remains controversial whether the consumption of high levels of dietary antioxidants can significantly increase the antioxidant capacity of humans (Bulb *et al.*, 2000; Pellegrini *et al.*, 2000).

Currently available biomarkers of plasma antioxidant capacity, such as the radical trapping antioxidant parameter assay (Wayner *et al.*, 1985) and the oxygen radical absorbance capacity (ORAC) assay (Gao and Prior., 1999) use hydrophilic radical generators, which produce radicals only in the aqueous compartment of plasma. However, plasma is made up of both aqueous and lipid compartments, and because antioxidants are either water soluble or lipid soluble, both the lipid and aqueous compartments should be monitored when assaying for the true total antioxidant capacity of plasma and for studying the influence that an individual nutrient or combinations of nutrients might have.

To control and reduce the free radical induced cellular damage, the organism has a compensatory mechanism, which comprises the most important variables in controlling or preventing free radical reactions. These defenses include some naturally occurring antioxidants as well as exogenous agents that have been proved useful. Some of these are water-soluble and some confined exclusively to non-polar environment such as ascorbic acid (vitamin C) and tocopherol (vitamin E) respectively. The other antioxidants

that have received maximum attention in biological systems include selenium and the thiol containing compounds like glutathione and the enzymes of glutathione cycle (Flohe, 1976; Kosower and Kosower, 1978).

2.1.1 Antioxidant vitamins

Antioxidative vitamins have a number of biological activities such as immune stimulation, inhibition of nitrosamine formation and an alteration of metabolic activation of carcinogens (Van Popel *et al.*, 1997). They can prevent genetic changes by inhibiting DNA damage induced by the reactive oxygen metabolites (ROMs) (Sun, Y. 1990). Lupulescu, (1996) showed that cancer cells synthesize an increased amount of DNA, RNA and proteins as compared to normal cells, which may be controlled by the administration of vitamins. An inverse association between β-carotene, α-tocopherol, and vitamin C, and breast cancer risk has also been reported (Ambrosone, 1995; Bohlke *et al.*, 1999). The major protective function of the vitamins against cancer is the scavenging of reactive oxygen metabolites (ROMS). (Torn *et al.*, 1995).

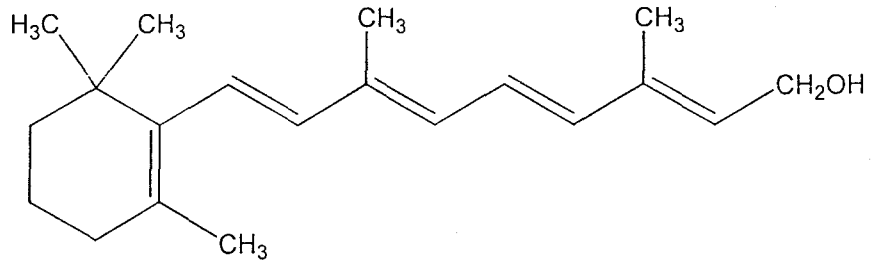
Mechanism of action as antioxidants

Two principle mechanisms of action have been proposed for antioxidants. The first is a chain-breaking mechanism, by which the primary antioxidant donates an electron to the free radical present in the system (e.g., lipid radical). The second mechanism involves removal of reactive oxygen species (ROS) / reactive nitrogen species (RNS) initiators (secondary antioxidants) by quenching chain-initiating catalysts (Ingold, 2003).

2.1.1.1 Vitamin A

Vitamin A is fat-soluble vitamin, which is essential for growth, maintenance of visual function, reproduction and differentiation of epithelial tissue. Vitamin A occurs mainly as the alcohol (retinol) in plasma and circulates as 1:1:1 complex with two hepatically synthesized proteins, retinol

binding protein (RBP) and transethyretin (or thyroxin binding pre-albumin). The amount in the circulation remains almost constant as the body stores decline during a period of deficiency, until the liver stores become too low to maintain this normal circulating level in the plasma. (Bates *et al.*, 1997).



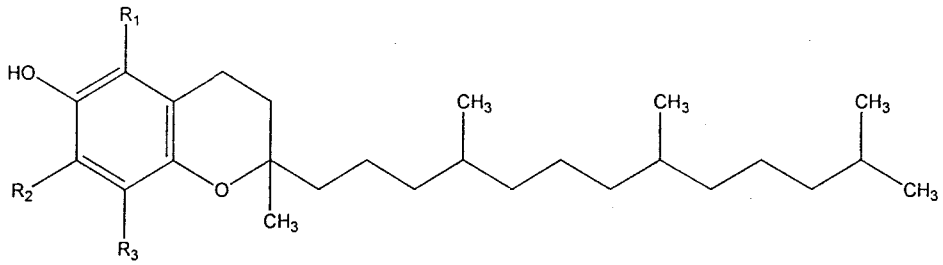
Structural formula of vitamin A

Vitamin A is reported to play a vital role in suppressing carcinogenesis by increasing immunity to tumors through several mechanisms. Vitamin A deficiency has been associated with a higher incidence of cancer and carcinogenesis (Tachibana *et al.*, 1995). The only specific effect of carotenoids in humans is to act as a source vitamin A in the diet, but they also have important antioxidant actions. The later are based on the carotenoids' ability to quench singlet oxygen and trap peroxyl radicals, thereby preventing lipid peroxidation. As a result, carotenoids protect against the development of cancer, cardiovascular disease, and ocular disorders (Polidori *et al.*, 2003). Carotenoids also affect cell growth regulation and gene expression. Diets low in carotenoids may lead to increased risk of cancer and heart diseases (Polidori *et al.*, 2003).

2.1.1.2 Vitamin E

Vitamin E is another lipid soluble vitamin. Vitamin E occurs in plasma as variety of tocopherols, of which the alpha and gamma isomers are usually the major ones. All vitamin E in the human body is derived from the diet. Major dietary sources of vitamin E are vegetable oils, margarine, nuts, seeds, whole grains and wheat germ. Different foods vary in the tocopherol (and

tocol) isomers; α-tocopherol is biologically most active isomer in mammals. Vitamin E circulates in the lipoproteins and chylomicrons, and the molar ratio to cholesterol is a good index of vitamin E status (Bates, 1997). Vitamin E is thought to be important in protecting the breast from free radical induced damage (London *et al.*, 1985). Because of antioxidant properties, vitamin E neutralizes reactive oxygen metabolites (ROMs) and reduces oxidative DNA damage and genetic mutation (Frei, 1994). Vitamin E is thought to be an important chain breaking antioxidant, which plays an important role in various stages of carcinogenesis through its contribution to immunocompetence, membrane and DNA repair, and decreasing oxidative DNA damage (Kimmick *et al.*, 1997). Vitamin E can directly act with a variety of oxy radicals including the peroxy radical ($ROO^{\bullet}$), $CCl_3^{\bullet}$, hydroxyl radical ($^{\bullet}OH$) (Mc Cay, 1985), oxy radical ($O_2^{\bullet}$) (Fukuzawa *et al.*, 1983) and singlet oxygen (Littarru *et al.*, 1984). Vitamin E donates hydrogen from the 6th position of its chromonal ring to free radical. The ROMs abstract H atom from the



Structural formula of vitamin E

PUFA in the cell membrane. The fatty acid radical formed reacts with oxygen that recycles to form more peroxy radicals in a chain reaction. The phenolic hydroxyl group of tocopherol reacts with an organic peroxy radical to form the corresponding organic hydroperoxides and the tocopheryl radical. It can reduce nitrite by inhibiting the production of carcinogenic nitrosoamines and nitrosomides (Garland *et al.*, 1993).

In vitro studies showed that vitamin E can prevent oxidation of DNA by inhibiting activated neutrophils (Van *et al.*, 1993). Vitamin E can protect the conjugate double bond of β-carotene from oxidation. The sparing action

of tocopherol on β-carotene was described *in vivo* in humans (Urbach *et al.*, 1997). Vitamin E can protect against many symptoms of selenium deficiency (Ganther *et al.*, 1976). These sparing as well as synergistic actions are thought to result from the ability of both tocopherol and selenium dependent glutathione peroxidase (GPx) to decrease the production of lipid peroxidation.

(vit E-O·): ROO. + vit E-OH ⟶ ROOH + vit E-O

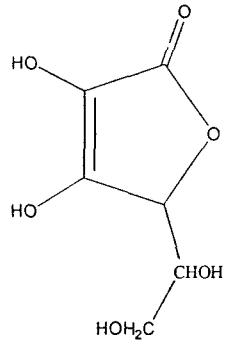
The chain of peroxidation reaction is effectively interrupted; the generated organic hydroperoxides can subsequently be detoxified via non-radical reaction. Tocopheroxyl radical can be reduced to tocopherol by interaction with reductants serving as hydrogen donors.

2.1.1.3 Vitamin C

Vitamin C (ascorbic acid) is an important water-soluble antioxidant in biological fluids and an essential micronutrient required for normal metabolic functioning of the body (Jaffe, 1984). Humans have no ability to synthesize vitamin C due to mutation in the gene coding for L-gulonolactone oxidase, an enzyme required for the biosynthesis of vitamin via the glucoronic acid pathway (Woodall *et al.*, 1997). Thus, vitamin C is obtained through diet. The vitamin is especially plentiful in fresh fruits, in particularly in citrus fruits and vegetables (Bendich, 1997). Vitamin C acts as a cofactor for several enzymes involved in the biosynthesis of collagen, carnitine and neurotransmitters (Burri *et al.*, 1997).

Vitamin C has been implicated for steroid metabolism in adrenals. Hydroxylation of aromatic drugs and carcinogens by hepatic cytochrome P_{450} is also enhanced by reducing agents such as vitamin C (Tsao, 1997). The temporal order of antioxidant consumption in human with blood plasma exposed to a constant flux of aqueous peroxy radicals is vitamin C > bilirubin > uric acid > vitamin E. Plasma devoid of vitamin C, but no other endogenous antioxidant, is extremely vulnerable to oxidant stress and susceptible to peroxidative damage to lipids (Frei *et al.*, 1989). Vitamin C readily scavenges ROMs, ozone, peroxynitrile radical ($ONOO^-$), nitric oxide (NO_2), nitric oxide

radical ($NO^{\bullet}$), and hypochlorous acid (Noorozi *et al.*, 1998). It has also been reported that vitamin C may enhance host immnunological functions (Kelly *et al.*, 1996). Epidemiological studies have indicated an inverse association between vitamin C intake and risk of cancers (Hecht , 1997). It can prevent carcinogenic nitrosamine formation in cancer, which is another protective function of vitamin C (Tannenbaum *et al.*, 1991). It can also protect lipid and lipoprotein against oxidative damage (Das *et al.*, 2005).



Structural formula of ascorbic acid (Vitamin C)

Vitamin C can act as a co-antioxidant by regenerating α-tocopherol from the α-tocopheroxyl radical produced during scavenging ROMs. In addition, vitamin C may reduce carcinogenesis through the stimulation of immune system, where cytotoxic T lymphocytes, macrophages and natural killer cells can lyse tumor cells (Bendich A, 1997). Vitamin C has also shown to regenerate urate, glutathione and β-carotene *in vitro* from their respective one-electron oxidation product, i.e. urate radicals, glutathyl radicals, and β-carotene radical cations (Edge *et al.*, 1997). Vitamin C may modulate the activity of hydroxymethylglutaryl-CoA reductase, the rate-limiting enzyme in the biosynthesis of cholesterol (Lynch *et al.*, 1996). Although vitamin C also reacts rapidly with hydroxyl radicals ($^{\bullet}OH$), it is nevertheless unable to preferentially scavenge this radical over other substrate (Niki *et al.*, 1997), because $^{\bullet}OH$ is extremely reactive and combines indiscriminately with any substrate in their immediate environment at a diffusion-limited rate (Carr *et al.*, 1997). Vitamin C may prevent the formation of (peroxynitrile) $ONOO^{-}$ by

reaction with singlet oxygen ($O_2^{\bullet}$) and may help to release nitric oxide radical ($NO^{\bullet}$) from endothelial cell by preventing the oxidation of LDL (Levine *et al.*, 1996). For this reaction high concentration of vitamin C is required because of large difference in rate constant.

Vitamin C may protect against carcinogenesis through several mechanisms in addition to inhibition of DNA oxidation. The oxidation of DNA is determined in cells and is found increased in case of oxidative stress, which is correlated with reduced plasma concentration of the antioxidative vitamins C and E (Fraga *et al.*, 1996). One potential mechanism is chemoprotection against mutagenic compounds such as nitrosamines. N-Nitroso compounds undergo activation by cytochrome P_{450}-dependent enzymes and have been implicated in gastric and lung cancer (Hemila, 1997). Vitamin C can protect host cells against harmful oxidants released into the extracellular medium. Vitamin C may also affect the production of immune proteins such as cytokines and antibodies as well as complement components (Hemila, 1997).

There are reports suggesting that $O_2^{\bullet}$ plays an important role in the oxidation of vitamin C (Ray *et al.*, 2001). The other study showed that production of singlet oxygen ($O_2^{\bullet}$) was significantly higher in breast cancer patients (Oshakhovitch *et al.*, 2004). Thus, higher $O_2^{\bullet}$ may be one of the possible causes of low plasma vitamin C in breast cancer patients. There are reports suggesting that vitamin C may prevent the formation of $ONOO^-$ by reacting with $O_2^{\bullet}$ and may help to release $NO^{\bullet}$ from endothelial cells (Fraga *et al.*, 1996). A decreased serum vitamin C level has been reported in colon, breast and stomach cancers (Ramaswamy *et al.*, 1996 and Block, 1991).

2.1.2 Makoi (*Solanum nigrum*)

Solanum nigrum Linn. (Solanaceae family) commonly known as 'Makoi', is a medicinal plant of 30-45 cm height, found throughout India up to 3000 m in the western Himalayas. Leaves are ovate or oblong, sinuate

tooted or lobed, narrowed at both ends, flowers are white and fruits are red, yellow or black. *S. nigrum* is used as stock for tomatoes to counteract the heat in North India. (Dhar, 1968). The herb has antiseptic and antidysentric properties and is given internally for cardialgia and gripe (Rastogi *et al.*, 1991). An infusion of the plant is used as an enema in infants having abdominal upsets. It is a household remedy for anthrax pustules and is applied locally. The plant is also credited with emollient, diuretic and laxative properties (Rastogi *et al.*, 1991).

2.1.2.1 Active constituents

Makoi plant contains a large number of biologically active constituents, some of these have been found to be extremely useful for treating various human and animal diseases. However, some plant constituents produce adverse effects on exposure. The onset of these adverse effects can be quite sudden to take sometime to develop. Fortunately among the thousands of plants in the environment of animals, relatively few cause acute illness when ingested.

The most important constituent of *S. nigrum* is solanine, which was discovered by Desfores, 1921. Different parts of the plant have been reported to contain steroidal alkaloid, glycosides, solasonine, α and β solamargine, as well as the steroidal sapogenins, tigogenins and diosgenin (Mallika *et al.*, 1976; Pkhedze *et al.*, 1967). The berries of *S. nigrum* have been recently studied and found to contain four steroidal glycosides, solamargine, solasonine, α and β solamargine all of which yield solasodine as a glycine (Varshney *et al.*, 1959). The fruits contain glucose and fructose (15-20%), vitamin C and β carotene, green unripe fruits however, contain glycoalkaloids and their eating is a toxic hazard to human beings as well as livestock. Immature green fruit of the plant contains four steroidal alkaloid (Singh *et al.*, 1965).

***Solanum nigrum* leaves**

The leaves of *S. nigrum* contain flavonoids, quercetin, 3-β-glucosyl, (1-6) β galactoside, quercetin-3-gentiobioside, quercetin, 3-galactoside. Several reports have indicated that leaves of *S. nigrum* contain moisture, protein, fats and carbohydrate. The leaves are rich source of riboflavin. The values for various vitamins present in the leaves (100 grams) are riboflavin 0.59; nicotine 0.91; vitamin C 11.0 mg. Higher values of vitamin C (20-40 mg / 100 g) have also been reported, leaves also contain β-carotene (0.74 mg / 100 gm), while citric acid is present to the extent of 5%. (Rao and Ramasastry, 1969).

2.1.2.2 Medicinal uses

Since ancient times, several plants and herbs have been used to treat gastrointestinal disorders, including peptic ulcers (Satyawati, 1987). A freshly prepared extract of the herb is effective in cirrhosis of liver (Useful plants, 1992). In Indian system of medicine *S. nigrum* is very commonly used as hepatoprotective agent (Jindal *et al.*, 1975; Saxena and Garg, 1979), which also affords protection against free radical-mediated hepatic damage (Krandikar *et al.*, 1963; Bardhan *et al.*, 1985).

In traditional medicine, the plant has also been used as a febrifuge, antidiarrheal, in eye diseases, and in hydrophobia (Athar *et al.*, 1992). The plant is also credited with emollient, diuretic and laxative properties and its decoction is regarded as an antispasmodic. Freshly prepared extract of plant is effective in the treatment of cirrhosis of liver and also serves as an antidote to opium poisoning (Rastogi *et al.*, 1991).

One of the early plant defense responses to pathogen or their elicitors is the production of reactive oxygen species (ROS) termed oxidative burst. The ROS production is assumed to play a key role in the integration of diverse strategies leading to disease resistance. On the other hand, cellular ROS accumulation should be strictly controlled, since they exacerbate cell damage. The fruits and leaves of *S. nigrum* have been reported to possess antihepatotoxic potential and inhibit lipid peroxidation by increasing free

radical scavenging enzyme activities (Nadeem *et al.*, 1997). The generation and subsequent involvement of free radicals in a large number of diseases such as myocardial ischaemia, carcinogenesis, inflammatory diseases, cataract formation and Alzheimer's disease have been recognized (Floyds, 1990; Fridrovich, 1988; Nakayma *et al.*, 1983). It has also been observed that proteins, lipids and DNA are major targets of oxidative injury (Athar *et al.*, 1987; Ames, 1989; Agarawal *et al.*, 1990).

2.1.3 Garlic (*Allium sativum*)

Historical perspectives of Garlic

Charaka-Samhita The leading surviving medical text has been associated with the healing process in India. Three ancient medical traditions i.e. Tibbi, Unani and Auryvedic, made extensive use of garlic as central part of the healing efficacy of plant (Moyers, 1996) and has recommended garlic for the treatment of heart diseases and arthritis (Woodward 1996); There are evidences suggesting the use of garlic extract for infections, worm infestations, weakness, fatigue, and in number of digestive disturbances.

The medical uses of garlic (*Allium sativum*) have a long history (Block 1985). Its use as a remedy for heart disease, tumors and headache are documented in Egyptian *Codex Ebers,* dating from 1550 BC. Garlic is mentioned in the Bible and has been a traditional treatment in many countries, notably the Near East, China and India. Apart from its medicinal uses, garlic is an integral component of diet. The effects of enzymatic synthesis of thromboxane in platelet ex vivo were reported after intraperitoneal administration of garlic (Ali and Mohammad, 1986).

2.1.3.1 Constituents of Aged Garlic Extract

Aged garlic extract (AGE) is an odorless product resulting from prolonged extraction of fresh garlic at room temperature; it is highly bioavailable and has a biological activity *in vivo* in both animals and humans (Moriguchi *et al.*, 1997). AGE contains water soluble allyl amino acid

derivatives which account for most of its organosulfur content; stable lipid-soluble allyl sulfides, flavonoids, saponins and essential macro and micronutrients (Amagase, 1998). The lipid-soluble volatile organosulfur compounds like allicin is produced enzymatically when garlic is cut or chopped. Allicin is an unstable and transient compound with oxidant activity (Freeman and Kodera 1995); it is virtually undetectable in blood circulation after gastric ingestion (Lawson *et al.*. 1992) because it decomposes to other organosulfur compounds (Freeman and Kodera, 1995).

The major unique organosulfur compounds in AGE are water-soluble S-allylcysteine (SAC) and S-allylmercaptocysteine (SAMC), which have potent antioxidant activity (Amagase. 1997; Ide and Lau, 1997; Imai *et al.*, 1994; Wei and Lau, 1998). The content of SAC and SAMC in AGE are high because they are produced during the process of aging, thus providing AGE with higher antioxidant activity than fresh garlic and other commercial garlic supplements (Imai *et al.*, 1994). Studies on the pharmacokinetics of SAC in a number of animal species showed that SAC is easily absorbed from the gastrointestinal tract and distributed in plasma, liver and other organs with a bioavailability of 98% in rats (Nagae *et al.*, 1994).

Lipid-soluble compounds in AGE include diallyl sulfide (DAS), triallyl sulfide, diallyl disulfide (DADS) and diallyl polysulfides (Amagase and Milner, 1993; Awazu and Horie, 1997; Horie *et al.*, 1992). The lipid-soluble organosulfur compounds also show antioxidant effect (Awazu and Horie, 1997, Horie *et al.*, 1992 and 1989).

The phenolic hydroxyl group of antioxidants in AGE like N-fructosyl glutamate, N-fructosyl arginine (O'Brein and Gillies, 1998), and allixin also confer antioxidant activity (Ide and Lau, 1997).

2.1.3.2 Medicinal uses

A substantial body of evidence showed that AGE and its compounds inhibit the oxidative damage that is implicated in a variety of diseases and aging (Imai *et al.*, 1994). These effects strongly suggest that AGE may have

an important role in lowering the risk of cardiovascular diseases, cancer, Alzheimer's disease and other age-related degenerative conditions, protecting human health and mitigating the effects of aging.

It has been reported that garlic also has a diuretic effect. It is possible that the mobilization of fluid from the extravascular space may have been due to improved cardiovascular function resulting from garlic treatment. Garlic is used as a potent antioxidant; it reduces blood pressure (Steiner *et al.*, 1996), improves elevated serum cholesterol (Rivilin, 1998) and decreases platelet aggregation (Steiner and Linn, 1999).

Recent studies have validated many of the medicinal properties attributed to garlic and its potential to lower the risk of diseases. It has been reported that garlic extract plays an important role in prevention of cancer in animals (Amagase and Milner, 1993; Milner, 1996; Nishimo *et al.*, 1990). Epidemiological studies show an inverse correlation between garlic consumption and reduced risk of gastric and colon cancer (Steinmertz *et al.*, 1994). The antithrombotic activity (Block, 1985), hypolipidemic and cardioprotective effect (Neil and Sigalic, 1994) of garlic have been ascribed to its potent antioxidant action (Wei and Lau, 1998; Yang *et al.*, 1993).

2.1.3.3 Antioxidant actions of AGE

Phytochemicals from plant-rich diets, including garlic, provide important additional protection against oxidant damage (Borek, 1997). The variety of antioxidant phytochemicals in AGE, which protect against disease-causing oxidative damage (Amagase, 1997; Horie *et al.*, 1992; Ide and Lau, 1997; Wei and Lau, 1998; Yamasaki *et al.*, 1991), may act in single and combined fashion (Amagase *et al.*, 1996; Borek, 1997 and 1993).

The antioxidant potential of AGE in scavenging reactive oxygen species and inhibiting lipid peroxide formation have been studied by various methods such as chemiluminescence (Imai *et al.*, 1994), *in vivo* inhibition of the release of pentane, inhibition of thiobarbituric acid reactive substance

(TBARS), a product of oxidized lipids, (Awazu and Horie, 1997, Amagase, 1997; Horie *et al.*, 1989).

AGE has been shown to inhibit lipid peroxide formation in several studies (Wei and Lau, 1998). TBARS induced by hydrogen peroxide were found inhibited 31-89% by AGE and 33-67% by SAC in a concentration-dependent manner (Yamasaki *et al.*, 1994), thus mitigating oxidant events, which are implicated in the formation of atherogenic lesions (Efendy *et al.*, 1997).

The antioxidant effects of allixin, SAC, SMAC and diallyl polysulfides have been reported (Imai *et al.*, 1994). Recently, spin resonance spectroscopy has revealed antioxidant role of other components of AGE, like N-fructosyl arginine and N-fructosyl glutamate (O'Brien and Gilles, 1998).

The exposure of bovine arterial endothelial cells to the oxidants, hypoxanthine, xanthine oxidase or hydrogen peroxide in the presence of AGE generated increased levels of SOD, catalase, GPx in a dose and time dependent manner, which suppressed the production of superoxide radical and hydrogen peroxide (Wei and Lau, 1998).

AGE and SAC have also been shown to prevent oxidant induced dense-body formation in sickled red blood cells (Lee *et al.*, 2000). The dense bodies are characteristic sickle cell anemia.

2.1.3.4 Toxic effects

Garlic possesses hypoglycemic and fibrinolytic activities (Bordia *et al.*, 1977; Kamanna *et al.*, 1982). The organic disulphide and sulphoxide could react with various SH group compounds in the body spontaneously at physiological pH and temperature. Capsules of garlic are widely used for its hypolipidemic effects and no serious side effects have been reported (Bobpoi *et al.*, 1984). The feeding of aqueous extract of garlic to young rats caused death of some rats due to severe stomach injury (Chaudhuri *et al.*, 1980), whereas the body weight of the surviving ones was considerably reduced due to less food and water intake. Hypertrophy of liver, spleen and adrenals,

followed by decreased erythrocytes count, are found the regular features in rats fed high dose of raw garlic (Banerjee *et al.*, 2002).

2.1.4 Onion (*Allium cepa)*

Several reports have suggested that garlic and to a lesser extent, onion have a protective effect against strokes, coronary thrombosis and atherosclerosis (Bordia *et al.*, 1977). Aqueous extract of onion has been shown to inhibit cyclogenase and lipoxygenase, as evidenced by reduced thromboxane and hydroxycosatetraenoic acid (HETE) formation from exogenous arachidonic acids (Srivastava, 1986 and 1984; Wanger *et al.*, 1990).

2.1.4.1 Active Constituents

Two sets of compounds make up the majority of onion's known active constituents-sulfur compounds, such as allyl propyl disulphide (APDS), flavonoids and quercetin. Each of the compounds has multiple medicinal actions.

The sulfur compounds form strongly scented oil, particularly the thioproanal-s-oxide or lachrymatory factor, which is responsible for the tearing many people suffer while cutting onions. (Brodnitz *et al.*, 1971). Onion and onion oil constituents have been repeatedly shown to kill various microbes in the test tube (Zohri *et al.*, 1995; Kim, 1997). But studies have not been conducted in humans to assess its antimicrobial action.

APDS has been shown to block the breakdown of insulin by the liver, and to stimulate insulin production by the pancreas, thus increasing the amount of insulin and reducing sugar level in the blood (Sharma *et al.*, 1977). Several uncontrolled human studies (Jain *et al.*, 1971; Methew *et al.*, 1975) and clinical trials (Tjokroprawiro *et al.*, 1983) have shown that large amount of onion can lower blood sugar levels in people with diabetes, but it does not reduce blood sugar levels in healthy non-diabetic people (Sharma *et al.*, 1977).

Garlic

Onion

2.1.4.2 Medicinal use

Onions (*Allium cepa* Liliacae) have long been used as a traditional remedy in the treatment of a variety of disorders. Researchers have reported pharmacological evidence for the use of onion as anthelmintic; anti-inflammatory; antiseptic; antispasmodic; carminative; diuretic; expectorant; febrifuge; hypoglycemic; hypotensive; lithontripic and anti-skin infective (Grieve *et al*, 1984). The onion is used in the offsets like angina, atherosclerosis and heart attack (Hertog et al., 1993). Baked onion can be used as a poultice to remove pus from sores. It has also been reported that onion is useful in preventing oral infection and tooth decay (Hertog et al., 1993). Although rarely used specifically as a medicinal herb, the onion has a wide range of beneficial actions on the body and when eaten (especially raw) on a regular basis will promote the general health of the body.

The anti-inflammatory effect of onion is strong enough as the subcutaneous injections and topical applications to inhibit skin reactions to intensely inflammatory compounds in people with or without eczema (Dorsch *et al.*, 1984). Human studies have not been performed to determine whether onion would be useful in people with asthma or cough, though the anti-inflammatory action cited above suggests it might be. These actions, coupled with an ability to reduce the stickiness of platelets (Chen *et al.*, 2000) and, overall, to decrease the thickness of the blood, (Kendler *et al.*, 1987) have led an interest in onion as a way to prevent or possibly reduce arteriosclerosis, though controversial results are reported for the use of onion in people with atherosclerosis (Kleijnen *et al.*, 1989). Intake of quercetin in the diet, primarily from onion, tea and apples, has been linked to a decreased risk of heart attack. (Hertog, 1983). High intake of quercetin and other flavonoids from onion and other foods have been shown to decrease risk of atherosclerosis in an epidemiologic study in the United States, although the result was not considered statistically significant (Rimm *et al.*, 1996). The clinical trials showed that a crude onion extract could lower blood pressure in some people with hypertension (Louria *et al.*, 1985). On the whole, it is

unclear whether or not onion supplements, as opposed to onions eaten as food, have a beneficial effect on heart disease. In a preliminary study of healthy male volunteers, administration of 50 grams of raw or boiled onion prevented the rise in serum cholesterol induced by consumption of a high-fat meal. (Sharma *et al.*, 1975).

The evidence on cancer prevention with onion suggests a benefit for some but not necessarily for all types of cancer. Onion consumption at a level of at least half an onion a day was associated with a 50% decline in stomach cancer risk. (Dorant *et al.*, 1996). Higher onion intake was also correlated with lower risk of breast cancer in a French epidemiological study (Challier *et al.*, 1998), while no protective effect against colorectal cancer was seen (Dorant, 1996).

Most human studies that have shown an effect from onions used at least 25 grams per day and often two to four times that amount (Tjokroprawiro, 1983; Jain *et al.*, 1971). Though some studies have found cooked onions acceptable, several studies suggested that cooking degrades onion constituents, fresh or raw onions are probably most active (Bordia, 1996; Tjokroprawiro, 1983; Chen *et al.*, 2000; Ali *et al.*, 1999). If a tincture, syrup, or oil extract is used, one tablespoon three times per day may be necessary for several months before effects are noted (Louria, 1985).

2.1.4.3 Toxic effects

Most people can eat onion in food without any difficulties. Higher intakes of onion may worsen existing heartburn, though it does not seem to cause heartburn in people who do not already have it (Allen, 1990). There are also isolated reports of allergy to onion, in some people with asthma (Valdivieso *et al.*, 1994), manifesting as skin rash and red, itchy eyes.

Onion is safe for use in children, and in small amounts in food, during pregnancy (though some pregnant women may have heartburn that onions could exacerbate) and nursing. It is unknown whether larger amounts of onion are safe during pregnancy and nursing. One study did find that baby rats

nursing from mothers that were fed onion developed a taste for onion and suffered no ill effects (Wuensch, 1978).

2.2 Stress

Life exists by maintaining a complex dynamic equilibrium, or homeostasis, that is constantly challenged by intrinsic or extrinsic forces or stressors, (Chrousos *et al.*, 1992). Stress a term set to mean the mutual action of forces that take place across any section of the body (Chrousos *et al.*, 1988) is a state of threatened homeostasis. The human body reacts to stress by activating a complex repertoire of behavioral and physiologic responses. Successful adaptive response can be specific to a stressor or can be relatively nonspecific, when a stressor of any kind exceeds a threshold magnitude. Alterations in the ability of the organism to respond to stressors, with the response being either excessive or inadequate in magnitude or duration, may lead to disease.

Stress responses of individuals are determined by a multiplicity of factors, several of them as quantitative genetics of human complex behaviors (Plomin *et al.*, 1994). It has been estimated that about two third of reliable variance in measured personality traits are due to genetic influence (Bouchard, 2001). Nevertheless development is an important modifier of the stress response, since it influences timing and strength of the counteracting forces. It is for this reason that infancy, childhood and adolescence, associated with increased biological dependency and physiological and psychological immaturity, entail increased vulnerability to maladaptive responses.

The adaptive response is characterized by both behavioral and physical changes. The former include increased arousal and alertness, heightened attention and suppression of sexual and feeding behaviors. The central component of the system constantly receives information from the higher centers of the CNS, the periphery and the environment of the peripheral actions. Thus, the stress system is an extremely complex, albeit highly

efficient and flexible, physiologic network that helps to coordinate the dynamic equilibrium of the organism.

The parvocellular corticotropin-releasing hormone (CRH) and arginine-vasopresin (AVP) neurons of the paraventricular nuclei (PVN) of the hypothalamus, along with the CRH neurons of the paragigantocellular and other nuclei of the medulla and other cell groups of the medulla are the central coordinators of the stress system, while the HPA axis and the efferent sympathetic/adrenomedullary system represent its peripheral limbs. (Chrousos *et al.*, 1992). The CRH and nonadrenergic neurons are stimulated by serotonin and acetylcholine, and inhibited by glucocorticoids, gama-aminobutyric acid (GABA), corticotropin (ACTH) and opioid peptides (Bagdy *et al.*, 1989).

Circulating ACTH is the key regulator of the glucocorticoids secretion by the adrenal cortex, but other hormones, some of them originating in the adrenal medulla participate as well. (Hinson, 1990). Glucocorticoids are the final effectors of the HPA axis and participate in the control of the whole body homeostasis and the organism's response. They also play a key regulatory role on the basal activity of the HPA axis and on the termination of the response by exerting negative feedback at the CNS components of the stress system (De Kloet, 1991).

Stress is known to induce alterations in various physiological responses even leading to pathological states (Chrousos, 1988). The stress-induced effects are supposed to be an outcome of altered activity of different mechanisms such as central neurotransmitters, neurohormonal factors, particularly those linked with the pituitary–adrenal axis, and free radical generation (Herman *et al.*, 1997). The majority of studies relating stress with neurotransmitters concern the biogenic amines (Hellgriel *et al.*, 1996). The cholinergic system, which has been proven to play an important role in the regulation of several central functions, however, has received less attention. There are some studies linking stress with cholinergic activity (Banu *et al.*, 1988)

Numerous clinical trials have been designed on the pathophysiological hypotheses identified in the experimental studies carried out in the previous years. ROS are involved in the development and progression of various cardiac diseases (Ferrari *et al.*, 1998) and oxidative stress as a pathologic determinant is a widely accepted concept. Unsaturated fatty acids in membranes, thiol groups in proteins, and nucleic acids are important targets in oxidative stress (Ceconi *et al.*, 2000).

Established risk factors such as hypertension, smoking, environment related diseases, etc., are all associated with increased oxidative stress due to excess ROS activity (Halliwell and Gutridge, 1989). When interest in oxidative damage started, unsaturated fatty acids appeared to be the likely initial oxidative target and lipid peroxidation appeared to be a reliable explanation for the dramatic structural changes of cellular membranes and the loss of ion homeostasis occurring upon post-ischemic reperfusion (Lucy, 1972; Ceconi *et al.*, 1988).

As a consequence of the convincing evidences obtained from experimental research and pathophysiological studies conducted in humans, it was thought that manipulation of oxidative stress has an effective improvement in the treatment of various disorders. Unfortunately, not a single pathophysiological achievement in the field of oxidative stress has had a significant impact on therapy so far.

2.2.1 Free radicals

Free radical is any species capable of independent existence that contains one or more unpaired electrons (An unpaired electron is one that occupies an atomic orbital or molecular orbital by itself). The presence of one or more unpaired electrons causes the species to be attracted slightly to a magnetic field, and makes the species highly reactive. Radicals can easily be formed when a covalent bond is broken, if one electron from each of the pair shared remains with each atom, a process known as *hemolytic fission*. Sources of O_2^- radical, such as photochemical or enzymatic system, have been

observed to inactivate enzymes, cause erythrocyte hemolysis, kill bacteria, degrade DNA, and destroy animal cells in culture.

Free radicals are of great importance in the mode of action of several toxic substances and in the process of inflammation (Chevian *et al.*, 1982; De Varies, 1981; Do Campo and Moreno, 1984). Radicals have been suggested to be involved in ischaemia and in degenerative arterial disease (Wilson, 1980).

2.2.1.1 Reactive oxygen species and their toxicity

Well over 90% of the O_2 that enters human cells is used for the production of energy by mitochondrial cytochrome oxidase, during the process; four electrons are added to each O_2 molecule resulting in the formation of two molecules of water.

$$O_2^- + 4H^+ + 4e^- \longrightarrow 2H_2O$$

For O_2 to oxidize, a molecule directly would have to accept a pair of electrons, and the electrons would have to have spins opposite to those of the unpaired electrons of O_2. The two electrons forming a covalent bond in a molecule would not meet these criteria since they have spins opposite to one another.

2.2.1.2 Superoxide anion radical

Within biological systems, O_2 usually accepts electrons one at a time resulting into the following intermediates.

$O^{\bullet}_2 \xrightarrow{e^-}$ superoxide anion radical ($O_2^{\bullet -}$)

$\xrightarrow{e^-}$ hydrogen peroxide (H_2O_2)

$\xrightarrow{e^-}$ hydroxyl radical ($^{\bullet}OH$) $\xrightarrow{e^-}$ (H_2O)

The intermediates have various degrees of reactivity with non-radical species. The acceptance of a single electron by O_2 generates the oxygen radical. The $O^{\bullet}_2$ is produced *in vivo* in a variety of ways. The major source of the reactant is via the electron chain in mitochondria (Nohl and Hegner, 1978; Sohal, 1997). It is generally held that $O^{\bullet}_2$ is not highly reactive towards

biological substrate in an aqueous environment. Moreover, once formed, $O^{\bullet}_2$ quickly undergo dismutation to generate H_2O_2, this reaction is markedly accelerated by a family of enzymes, the superoxide dismutases (SOD) (Fridovich, 1989). Since SOD removes an oxidant, that is, $O^{\bullet}_2$ from the cell it is generally considered an important antioxidative enzyme with the hydroperoxy radical (H_2O_2).

$$O^{\bullet}_2 + H^+ \longrightarrow HO^{\bullet}_2$$

Under conditions of tissue acidosis, which can occur in the nervous system during ischaemia, it favors the formation of peroxy radical ($HO^{\bullet}_2$).

$HO^{\bullet}_2$ is much more lipid soluble and is far more powerful oxidizing or reducing agent than $O^{\bullet}_2$. Thus, in an acidic environment lipid peroxidation due to the conversion of $O^{\bullet}_2$ to $HO^{\bullet}_2$ is greater. Finally, $HO^{\bullet}_2$ has a much higher rate of dismutation to H_2O_2 than $O^{\bullet}_2$.

2.2.1.3 Hydrogen peroxide

Besides SOD, several other enzymes like L-amino acid oxidase, glycolate oxidase and monoamine oxidase generate H_2O_2 and exist in human tissues (Masini-Repiro *et al.*, 2004). In dopaminergic nerve terminals the oxidative deamination of dopamine (DA) by monoamine oxidase is the chief catabolic pathway (Cohen, 1988). It has been proposed that the accelerated turnover of dopamine (DA) in the brain of Parkinson's patients may account for the increase oxidative stress in dopaminergic terminals, which eventually leads to their destruction. Likewise, the side effects of prolonged L-DOPA treatment in Parkinson's patients may also be related to excessive H_2O_2 formation and its conversion to more highly toxic molecules (Olanow, 1990).

H_2O_2 itself is not equally toxic unless it is in high concentration within cells. H_2O_2 readily diffuses through cellular membranes and can thereby distribute to sites distant from where it was generated. Also, in the presence of transition metals, most often Fe^{2+} but also Cu^+, H_2O_2 is reduced to $^{\bullet}OH$ via either the Habers Weiss or Fenton reactions (Imlay *et al.*, 1988; Halliwell and

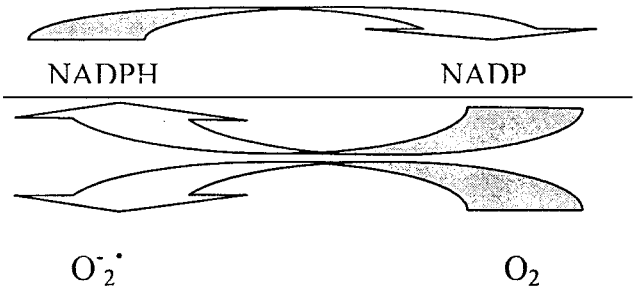
Gutridge, 1990). The ultimate fate of H_2O_2, however, is not always $^{\bullet}OH$. In most cells H_2O_2 is converted into innocuous products by the action of two important antioxidative enzymes, that is catalase and selenium dependent glutathione peroxidase (GPx). In the tissues the GPX are considerably more important than catalase because of the low activity of the latter enzyme in most parts of CNS (Jain *et al.*, 1991). GPx utilizes H_2O_2 and hydrogen peroxides as substrate during the conversion of reduced glutathione (GSH) to its disulphide (GSSG) (Griffith, 1985).

2.2.1.4 Hydroxyl radicals

The hydroxyl radical is perhaps most destructive species that is formed during the Fenton reaction (Bielski, 1991) and its formation is well documented to oxidize adjacent molecules (Halliwell and Gutteridge, 1989). An intermediate in the reaction of H_2O_2 with Fe^{2+} may be the iron oxygen complex referred to as ferryl which itself is highly oxidizing and which degrades to form the $^{\bullet}OH$. Once formed, the $^{\bullet}OH$ reacts rapidly with any molecule within a few angstroms of its site of production. Because of its high reactivity its estimated half-life at 37^0C is of the order of 1×10^{-9} sec. The $^{\bullet}OH$ readily damages nuclear and mitochondrial DNA, membrane lipids and carbohydrates. When the $^{\bullet}OH$ is produced within the mammalian cells it always damages DNA (Cochrane, 1991).

There are at least two ways in which DNA damage is achieved. In many cases the mutilated DNA may occur because H_2O_2 reacts with either Fe^{2+} or Cu^{+} that is bound to these two molecules in the immediate vicinity of DNA, such that when the toxic $^{\bullet}OH$ is formed its target is the adjacent nucleic acid (Halliwell and Arouma, 1988). Alternatively, during excitatory neurotransmitter stimulation of neurons, the large increase in the intracellular free Ca^{+} activates nuclease enzymes in nucleus, which results in the formation of $^{\bullet}OH$, which subsequently leads to DNA damage (Orrenius *et al*, 1989).

Hydroxyl radical also interacts with membrane lipids to initiate lipid peroxidation, this is accomplished when $^{\bullet}OH$ removes allelic H^+ from a PUFA, and this results in a radical chain reaction wherein lipid peroxidation is self-propagating. The process of lipid decomposition is favored during ischemic acidosis. During neural Ischaemia for example, lipid peroxidation could actually be initiated in the absence of an oxygen radical initiator when iron released from storage proteins reacts with lipid hydroperoxides, thereby decomposing them to peroxyl and alkyl radicals which can abstract a H^+ leading to further lipid peroxidation (Halliwell and Gutridge, 1990). The brain is preferred site for lipid peroxidation because of its regionally high content of iron and due to the fact that neural membrane phosphor-lipids are composed of a high content of easily oxidizable PUFA such as linoleic acid (18:2) and arachidonic acid (20:4).



Then

$$O^-_2{}^{\bullet} + O^-_2{}^{\bullet} \xrightarrow{2H+} H_2O_2$$

$$Fe^{3+} + O^-_2{}^{\bullet} \longrightarrow Fe^{2+} + O_2$$

$$Fe^{2+} + H_2O_2 \longrightarrow {}^{\bullet}OH + OH^- + Fe^{3+}$$

H_2O ← GPx: GSSG ⟷ GSH (GR)

Lipid peroxidation
Cell Death

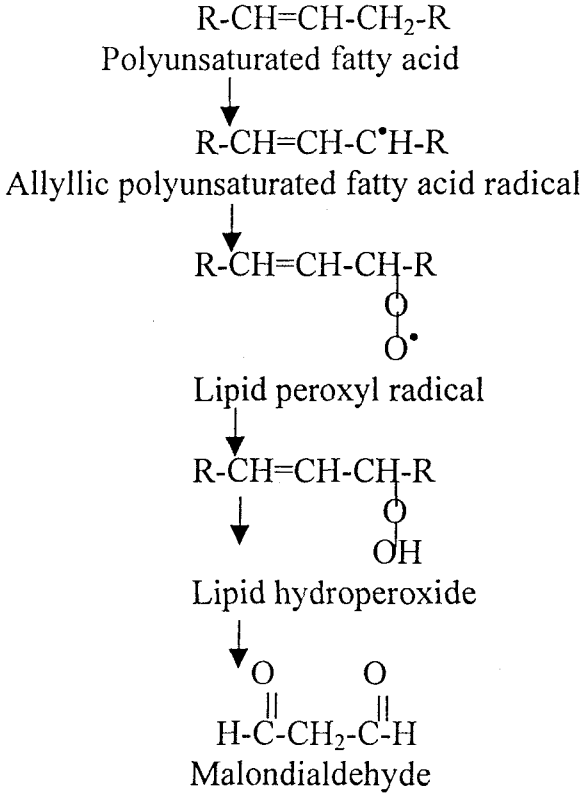
2.2.1.5 Singlet oxygen

Singlet oxygen is formed under photo-oxidative conditions when energy is transformed to O_2 from photo-excited sensitizers, like rose bengal or methylene blue. This is not a di-radical like molecular oxygen and it is highly

reactive towards most olefins; thus, it can abstract a H^+ from PUFA to initiate lipid peroxidation. In biological systems, O_2 may play a prominent role in the peroxidation of membrane lipids (Hailiwell and Gutridge, 1989).

2.2.1.6 Peroxyl radicals

The most thoroughly studied of all oxidative processes is that of the break down of lipids in cellular membranes during which the peroxyl radical is formed (Asano *et al.*, 1991). The process, referred to as lipid peroxidation, is extremely complex and can be self-propagating which means that once initiated it would theoretically lead to the oxidation of all the lipids in a cell. Thus, it can be highly destructive. However, the $^{\bullet}OH$ is not the only radical that can begin the process of lipid destruction; O_2 and $OONO^-$ can also do (Sevanian and McLeod, 1997).



Once underway, a number of toxic products are generated during the decomposition of fatty acids. These include lipid hydroperoxides and the peroxyl radical. They can attack a nearby PUFA and propagate the process.

Vitamin E is the premier peroxyl radical scavenger and chain breaking antioxidant (Packer, 1993).

2.2.1.7 Nitric oxide and peroxynitrite anion

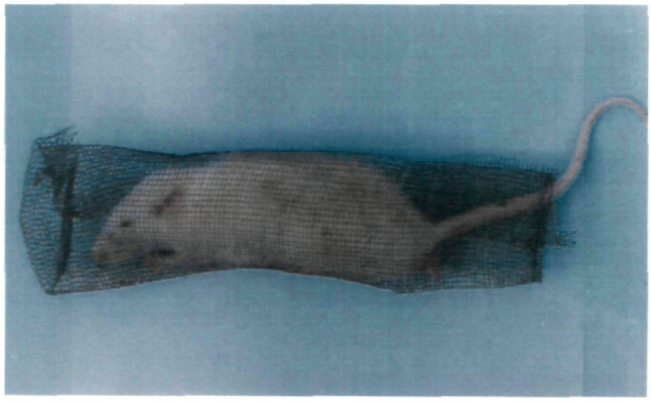
Nitric oxide radical ($NO^{\bullet}$) is often characterized as a double-edged sword. Under the normal physiological conditions the nitrogen centered radical has important function as a neuronal messenger molecule; however, when $NO^{\bullet}$ increases intra-cellularly to unusually high concentrations it initiates a toxic cascade of events, which can lead to the death of neurons (Dawson *et al.*, 1992). A common example of $NO^{\bullet}$ toxicity is seen in glutamate neurotransmission in the CNS where N-methly-D-aspartate (NMDA)-receptor activation leads to a large rise in Ca^{2+} followed by the stimulation of neuronal nitrogen oxygen species (NOS) (Dawson *et al.*, 1991), leading to the generation of $NO^{\bullet}$, this induces a series of events that can lead to neuronal destruction. During focal Ischaemia events in the CNS the release of excitatory amino acid neurotransmitters including glutamate large increases in $NO^{\bullet}$ are observed.

2.3 Immobilization stress and its pro-oxidant effect

It is well known that immobilization stress induces formation of reactive oxygen species (ROS) and leads to the oxidative injury in various tissues (De Castro *et al.*, 2000; Nishida *et al.*, 1997; Toleikis and Godin, 1995). The involvement of free radicals and free radical reactions have been observed in the etiology and development of a number of diseases, especially life limiting (Pyror, 1994). Role of reactive oxygen species have been reported in some emotional stress models (Guhaeva *et al.*, 1988; Deviatkina *et al.*, 1989) and in oxidative stress related diseases (Sies, 1991). Oxidative damage to lipid protein and DNA in the brain has been observed during immobilization stress (Liu *et al.*, 1996).

Oishi and Machida (2002) have shown that a significant increase in plasma thiobarbituric acid reactive substances (TBARS) was observed during

Immobilization Cage



Immobilized Rat

and after the stress. The activation of immune cells can be a source of the stress-induced ROS production and antioxidant enzymes in immune cells play an important role in preventing the ROS-induced injury (De Castro *et al.*, 2000; Babior, 2000; Victor *et al.*, 2003).

Oxidant-antioxidant balance is critical for immune cell functions because of its protective effect of the maintenance of cell membrane integrity and functionality (Pawlak *et al.*, 1998a, 1998b; Knight, 2000; Celada and Nathan, 1994). Stress is accepted as a response of an organism to external stimuli (Dorshkind and Horseman, 2001; Peng *et al.*, 2000). Stress has been shown to suppress different immune parameters, e.g. delayed type hypersensitivity, antibody production, NK activity, leukocyte proliferation, skin homograft rejection, and virus-specific T cell activity (Dorshkind and Horseman, 2001; De Castro *et al.*, 2000; Dhabhar, 2000) thus causing immunosuppression. Immune cells are particularly sensitive to oxidative stress because of the presence of polyunsaturated fatty acids in their plasma membranes and production of ROS, which is a part of their normal function (Pawlak *et al.*, 1998a; Knight, 2000; Celada and Nathan, 1994). Moreover, membrane-related functions are critical in maintaining normal function of immune cells and their ability to defend against foreign antigens (Biselli *et al.*, 1996; Yuli *et al.*, 1982). These functions are highly sensitive to ROS (Babior, 2000; Victor *et al.*, 2003).

2.3.1 Free radical metabolizing enzymes and other parameters

Free radical reactions are ubiquitous in living beings because of the high chemical reactivity of the intermediates. Various pathways are known by which free radicals can mediate cellular toxicity. The action of free radicals on biological system has the potential for disturbing the balance of pro-oxidants and anti-oxidants. An alteration in this balance in the favor of pro-oxidant is known as oxidative stress (Sies, 1985). Antioxidants are a group of substances which, when present at low concentrations, in relation to oxidizable substrates, significantly inhibit or delay oxidative processes, while

This is analogous to the dismutation of hydrogen peroxide to oxygen and water catalyzed by catalase, electrostatic repulsion between two molecules of superoxide anion limit their approach to one another, SOD overcomes the barrier and greatly increases the dismutation rate (Fridovich, 1978, 1976).

Several forms of SOD have been identified since McCord and Fridovoch first discovered the enzyme in 1971. They identified the enzymatic activity associated with erythrocuprein, a copper-zinc protein of erythrocytes. The copper is associated with enzymatic activity, whereas the zinc is structural. Similarly, SOD activity is associated with a family of Cu-proteins, cerebrocuprein in brain and hepatocuprein of liver (Fried, 1979). In mammalian tissues, a second form also exists in which manganese is the prosthetic group (Fridovich, 1979). In rats and mice the Mn SOD is localized in mitochondria, whereas Cu-Zn SOD is cytoplasmic. However, the distribution does not hold in other species.

SOD in various tissues of rats appears to protect against the toxic effects of oxygen free radical generated by its further reaction with cellular component (McCord, 1971; Fridovich, 1975). Fried and Mandel (1975) indicated that very high levels of SOD activity are present in liver, while the adrenals, kidney and red blood cells have intermediate activity and lower activities were found in other tissue including brain. It is the first enzyme of scavenger series to ameliorate the damage caused in the cells by free radicals (Slatter, 1984). Singlet oxygen and superoxide radical are potentially toxic to living cells as they can participate in the oxidation of cell macromolecules like protein, lipids etc in case of leak from the original oxidation reaction (King, 1975). Superoxide anions are generated during interaction with molecular oxygen with flavins, NADH, glutathione peroxidase and catecholamines (Misra and Fridivich, 1972; Heikkila and Cohen, 1973). Immobilization stress induces antioxidant defense changes in the plasma of rats (Liu *et al.*, 1994). Several workers have reported the role of oxygen free

radical in the restraint stress induced gastric lesions and the role of SOD in clinical studies on stress gastritis and prophylaxis (Kayabeli *et al*, 1994).

2.3.1.2 Catalase (EC 1.11.1.6)

Most purified catalases have been shown to consist of four protein subunits, each of which contains a haem (Fe (III)- protophorphyrin) group bound to its active site. Dissociation of the molecule into its subunits, which easily occurs on storage, freeze-drying, or exposure of the enzyme to acid or alkali, causes loss of catalytic activity. The three dimensional structures of catalase from beef liver and the fungus *Penicillium vitale* have been determined by X-ray crystallography.

It exists to remove hydrogen peroxide within cells, and catalyzes the reaction:

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

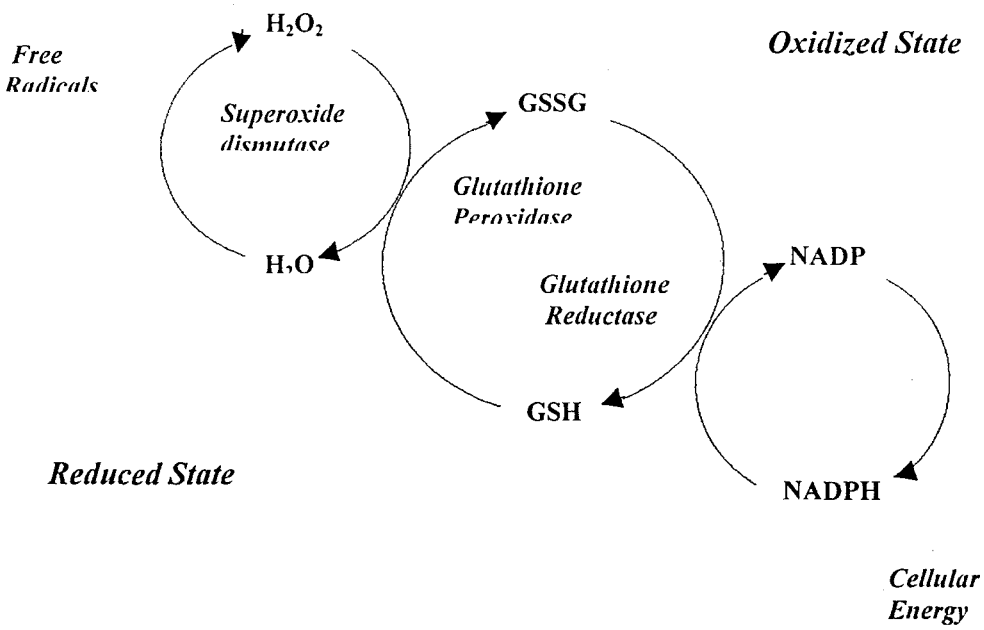
Immobilization stress was found to decrease the activities of catalase and glutathione peroxidase in rats (Kostic *et al.*, 2000; Al-Qirim *et al.*, 2002).

2.3.1.3 Glutathione

Glutathione is a tripeptide and is most abundant intracellular thiol often attaining millimolar levels inside the cells, which makes it one of the most highly concentrated intracellular antioxidants. It is a water phase antioxidant and is ubiquitous in microbes, plants, animals, and mammalian tissues and being water-soluble is found mainly in the cell cytosol and other aqueous phases of the living system (Kosower and Kosower, 1978). Glutathione has a high electron donating capacity and a high negative redox potential. This property of glutathione is of critical importance and is used in conjunction with other antioxidants to conserve the entire spectrum of biomolecules, to regulate their function and to facilitate the survival and optimal performance of the cell as a living unit. A healthy cell maintains greater than 90% of its glutathione in the reduced GSH form, which is an electron-rich antioxidant. The oxidized form (GSSG) is relatively electron poor. Thus, intracellular

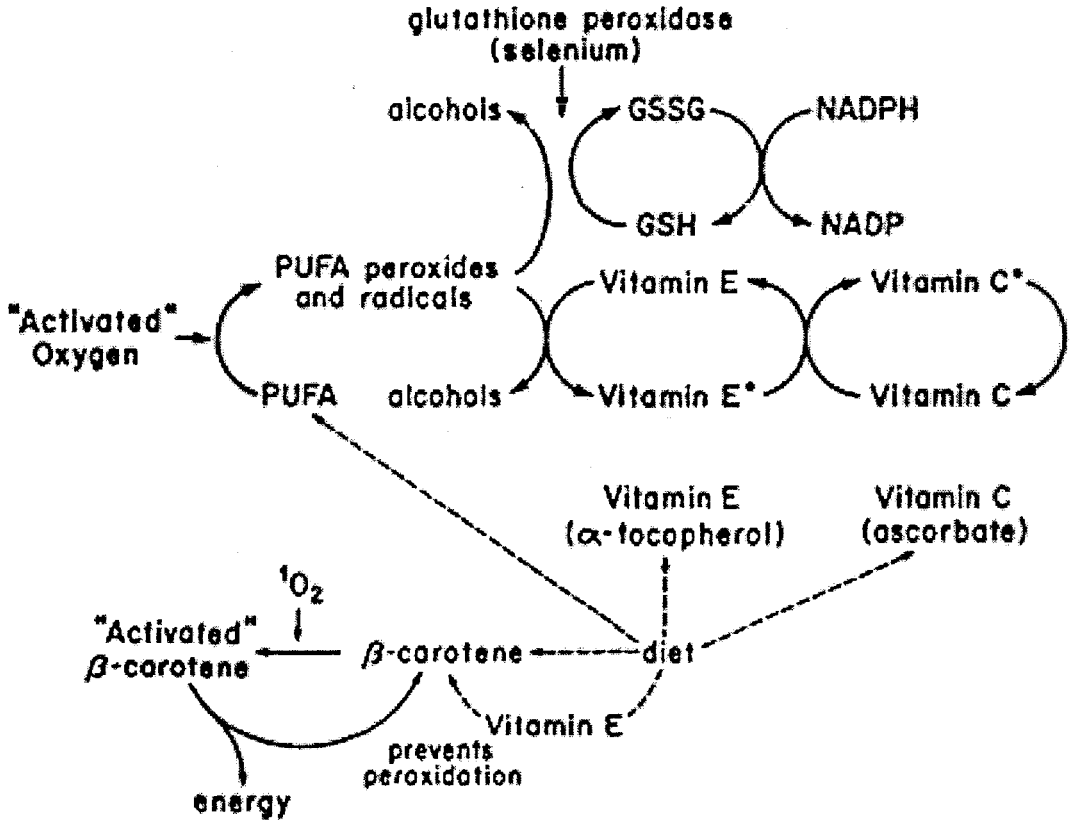
GSH status appears to be sensitive indicator of the cell's overall health, and of its ability to resist toxic challenge. This implies that intracellular GSH/GSSG ratio may be a sensitive indicator of oxidative stress.

Glutathione has been considered to function as a biological antioxidant. Its role in the destruction of free radicals has been observed (Sohal *et al.*, 1984 and Milner *et al.*, 1993). Several workers have also reported the role of glutathione in cellular protection during aging (Pruche *et al.*, 1991). Combined action of glutathione and superoxide dismutase (SOD) forms an integral component of cellular antioxidant defense (Munday and Winterborn, 1989).



Enhanced level of lipid peroxides along with depletion of glutathione has been observed in oxidative stress (Younesa and Sienger, 1980; Katoh *et al.*, 1989). Glutathione inhibits the oxidative stress induced by different compounds like ascorbate, NADPH-$BrCC_3$ and NADPH-Fe^{++} (Wafers and Sies, 1988; Tampo and Yanaha, 1990). It plays a protective role in rats against toxic oxygen species generated by hypoxia (Van *et al.*, 1985). Glutathione is essential for the repair process in brain exposed to oxidative damage by free radicals (Pellmar *et al.*, 1992). Depletion of glutathione during immobilization

stress stimulates oxidant and oxidative damage leading to degenerative diseases of aging including brain-dysfunction (Liu *et al*, 1996). Some workers have implicated the loss of antioxidant glutathione in the pathogenesis of Parkinson's disease (Drukarch *et al.*, 1996).



2.3.1.4 Glutathione-S-transferase (EC 2.5.1.1.18)

Glutathione-S-transferase is a non-selenium dependent glutathione peroxidase (Sies *et al.*, 1979). It was first identified in 1961 (Booth *et al.*, 1961; Coombs and Stakelum, 1961). The enzyme was subsequently named glutathione-S-transferase. Later on, several other GSTs were demonstrated depending upon the substrate specificity. Following types of GSTs have been described so far.

i. Glutathione-S-transferase, catalyzing the conjugation of a variety of alkylhalides with glutathione (Johnson, 1966).

ii. Glutathione-S-epoxide transferase, active towards conjugation of variety of alkylhalides with glutathione (Boyland and Williams, 1965).

iii. Glutathione-S-alkene transferase, catalyzing the conjugation of unsaturated compounds with glutathione.

The enzymes are almost ubiquitous in nature, and GST has been identified in man, non-human primates, rats, mouse, hamster, guinea pig, chicken, cow, sheep, trout, and shark (Mannervik, 1985). The concentration of GST, in general is high in mammals (up to 10% of cytosolic proteins in some organs), in other species (shark) the level of activity is quite low (Suguyama *et al.*, 1981). In addition, it is generally present in most mammalian organs.

Glutathione-S-transferase (GST) was found reduced in rats with enervation of the liver, thus confirming the role of the peripheral nervous system (Spiridonov *et al.*, 1989). It has a major role in the detoxification of oxyradicals and their products (Mannervik and Danielson, 1988). Brain GST plays an important role in the detoxification of potential toxicants through their conjugation and biotransformation (Booth *et al*, 1961; Boyland and Chasseaud, 1969; Dixit *et al.*, 1980; Kubota *et al.*, 1985). Greater accumulation of the toxic compounds inhibits the GST activity (Boyland and Chasseaud, 1969). GST has also been reported as tumor marker enzyme for detection of initiated cells during liver carcinogenesis (Tatematsu, *et al.*, 1988).

2.3.1.5 Transaminases (Aminotransferases)

The transaminases constitute a group of enzyme that catalyzes the interconversion of amino acid and α-ketoacid by transfer of amino group. The α-ketoacid glutarate/L-glutamate couple serves as an amino group acceptor and donor pair in all amino group transfer reactions, the specificity of the individual enzymes derives from the particular amino acid that serves as the other donor of an amino group.

Aspartate transaminase (GOT; EC. 2.6.1.1) has been isolated from the thermophilic microorganism *Bacillus isothermophillus* (Bartsch Klaus *et al.*, 1996). In viral hepatitis and other form of liver diseases associated with hepatic necrosis, serum GOT and GPT (EC. 2.6.1.2) are found elevated even before the clinical signs and symptoms of disease appear (e.g jaundice). Five to ten fold elevations of the two enzymes occur in patients with primary or metastatic carcinomas of liver, with GOT usually being higher than GPT (Vinitha *et al.*, 1995). Rapid increases in the activities of the two enzymes in serum have been reported during restraint stress. (Sun *et al.*, 1995) and slight or moderate elevations of both SGOT and SGPT activities may be observed after intake of alcohol and after administration of a variety of drugs like ampicillin (Novert, 1996).

Role of free radicals in stress diseases

A plausible explanation for the association of age and disease is based on the implication of free radical reactions in the pathogenesis of several disorders. Free radical reactions are expected to produce progressive adverse changes that accumulate with age throughout the body. Such "normal" changes with age are relatively common to all. However, superimposed on this common pattern are influenced by genetics and environmental differences that modulate free radical damage. These are manifested as diseases at certain ages determined by genetic and environmental factors. Cancer and atherosclerosis, of the two major causes of death, are salient "free radical" diseases. Cancer initiation and promotion is associated with chromosomal defects and oncogene activation.

It is possible that endogenous free radical reactions, like those initiated by ionizing radiation, may result in tumor formation. The highly significant correlation between consumption of fats and oils and death rates from leukemia and malignant neoplasia of the breast, ovaries and rectum among persons over 55 years may be a reflection of greater lipid peroxidation (Palmer *et al.*, 2004). Studies on atherosclerosis reveal the probability that the

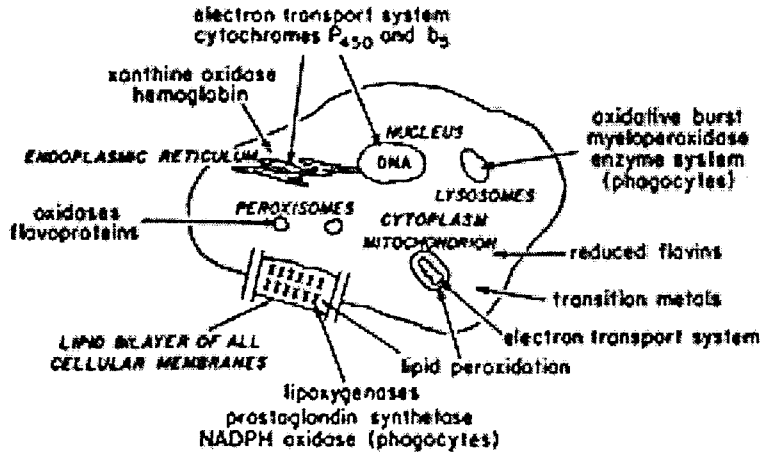


Figure 1. Cellular sources of free radicals

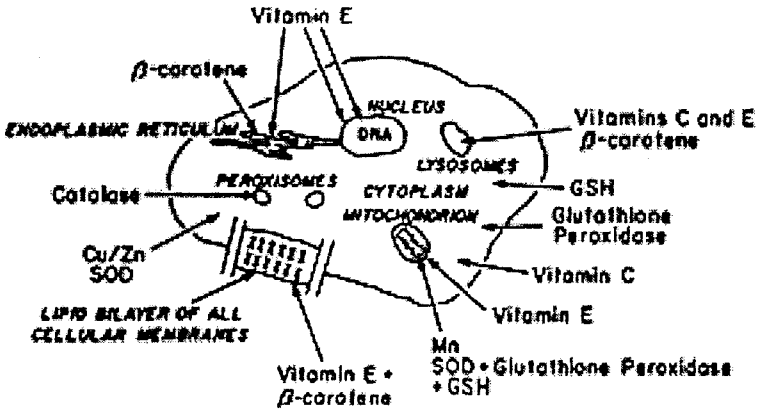
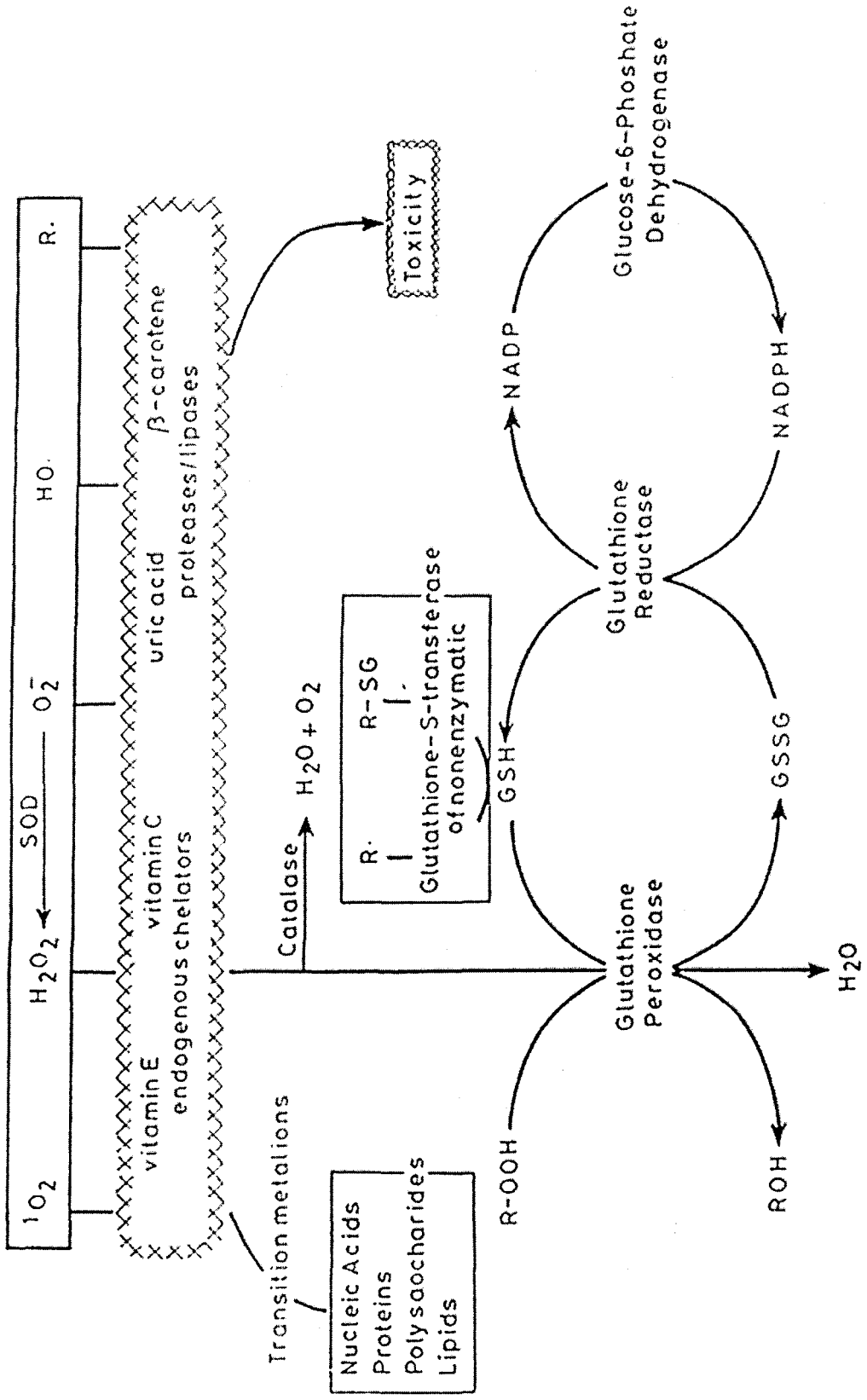


Figure 2. Antioxidant potential within cell

disease may be due to free radical reactions involving diet-derived lipids in the arterial wall and serum to yield peroxides and other substances. These compounds induce endothelial cell injury and produce changes in the arterial walls (Zhao, 2004).

Free radicals, however, are not always harmful. They also serve useful purposes in the human body. Several observations indicate that the oxygen radicals in living systems are probably necessary compounds in the maturation processes of cellular structures. Furthermore, white blood cells release free radicals to destroy invading pathogenic microbes as part of the body's defense mechanism against disease. Hence, the complete elimination of these radicals would not only be impossible, but also harmful.

Immobilization/restraint stress is an easy and convenient method to induce both psychological (escape reaction) and physical stress (muscle work) resulting in restricted mobility and aggression (Ramanove *et al.*, 1994; Singh *et al.*, 1993). Recently various stresses have been associated with enhanced free radical generation causing oxidative stress (Sies, 1983).



Biological antioxidant defense system

MATERIALS & METHODS

3. EXPERIMENTAL

3.1 MATERIALS

1-chloro-2, 4-dinitro-benzene (CDNB)	Sigma Chemical Co, USA
5-5′ dithiobis-2-nitrobenzoic acid (DTNB)	Sigma Chemical Co, USA
Acetic acid	Sisco Research Laboratory, Mumbai
Acetlythiocholine Iodide	Sisco Research Laboratory, Mumbai
Acetone	Qualigens, Banglore
Alkaline phosphatase	Sisco Research Laboratory, Mumbai
Bovine Serum Albumin (BSA)	Sisco Research Laboratory, Mumbai
Chloroform	Sisco Research Laboratory, Mumbai
Copper Sulphate	Qualigens Laboratories, Banglore
Ethylenediamine tetra-acetic acid (EDTA)	Ranbaxy Laboratory, Mumbai
Folin's Reagent	Sisco Research Laboratory, Mumbai
Glutathione	Sisco Research Laboratory, Mumbai
Glycine	Qualigens Laboratories, Banglore
Hydrochloric acid	Qualigens Laboratories, Banglore
Hydrogen peroxide	Qualigens Laboratories, Banglore
Methanol	Qualigens Laboratories, Banglore
n-Butanol	Qualigens Laboratories, Banglore
p-nitrophenol phosphate	Sisco Research Laboratory, Mumbai
p-nitrophenol	Sisco Research Laboratory, Mumbai
Potassium buffer component	Qualigens Laboratories, Banglore
Potassium Chloride	Qualigens Laboratories, Banglore
Potassium Sodium Tartarate	Qualigens Laboratories, Banglore
Pyrogallol	Qualigens Laboratories, Banglore

Sodium Azide	Qualigens Laboratories, Banglore
Sodium bicarbonate	Qualigens Laboratories, Banglore
Sodium buffer component	Qualigens Laboratories, Banglore
Sodium carbonate	Sisco Research Laboratory, Mumbai
Sodium chloride	Qualigens Laboratories, Banglore
Sodium dodecyl salicylate	Sisco Research Laboratory, Mumbai
Sodium hydroxide	Sisco Research Laboratory, Mumbai
Succinic Acid	Qualigens Laboratories, Banglore
Thiobarbituric acid (TBA)	Sisco Research Laboratory, Mumbai
Tri-chloro acetic acid (TCA)	Qualigens Laboratories, Banglore
Tris-hydroxy amino methane (Tris)	Sisco Research Laboratory, Mumbai
MDA Standard	Sisco Research Laboratory, Mumbai

3.2 Methods

Experimental animals

For the present study male Albino Wistar strain rats weighing 180-200 gm were selected, the animals were housed in-group cages; Purina diet and tap water were supplied to them ad libitum. Prior to the commencement and throughout the experiment the rats were housed at 24 ± 3^0C room temperature and 12 h dark / light cycles. All the experimental protocols adhered to the guidelines of animal welfare committee of the university.

3.2.1 Controls

Control animals were handled at the same time as the other experimental animals and were placed in individual cages during the corresponding time. Two types of controls were included. One who did not receive anything orally and served as controls for stress alone treatments, the other received olive oil / n-saline serving as controls for vitamin and garlic / onion, *Solanum nigrum* extracts treatments respectively. All the oral treatments to the rats were given through catheter.

After 1 hr of the termination of the experiment animals were sacrificed by injecting sodium pentobarbital (i.p 50 mg/kg body weight) and heparinized blood was collected and was separated by centrifugation at 5000 rpm for 10 min and was subjected for the assay of SOD, GST, catalase, MDA, ALP, SGOT, SGPT, glucose, uric acid, total and free SH groups. Tissues like brain, liver, kidney, spleen and heart were taken out from each rat, washed with chilled normal saline, homogenized in 0.1 M phosphate buffer pH 7.4 and kept at –20^0C till used.

3.2.2 Immobilization stress

Immobilization stress was accomplished by placing the individual animals in wire mesh cages of their size attached to a wooden board (Singh et al., 1993). The rats were deprived of food and water during stress exposure.

Rats were immobilized for a period of 2, 4,6,8,10,12 and 24 hrs by the standardized method (Singh et al., 1993). For further studies to evaluate the effect of vitamins A, E, C and various extracts, the animals were exposed to 6 hrs of stress.

3.2.3 Vitamin treatment

The effect of oral administration of vitamin A, E and C (15mg/kg body weight in water and olive oil respectively) alone and in combination (vitamins E+C) (15 mg/kg of body weight in 1ml of olive oil) was evaluated in rat plasma and various tissues.

The effect of high dose of fat-soluble vitamins A and E (50 mg/kg of body weight in 1 ml of olive oil) was also seen on biochemical parameters like SOD, GST, catalase, MDA, ALP, GOT, GPT and glutathione.

Vitamin and stress treatment

Both the prophylactic and possible curative roles of the antioxidant vitamins were evaluated on restraint stress induced oxidative changes.

Pre-treatments of vitamin

For the study of the pre-vitamin stress treatment, animals were divided into four groups (10 rats each), the first three groups received a single oral dose of vitamin A, E dissolved in olive oil and C dissolved in water (15 mg/kg body weight) individually and the fourth group received the same dose in combination (Vit E+C) prior to 6 hrs of immobilization stress.

Another set of rats received single high dose of Vitamin A or E (50 mg/kg body weight) individually dissolved in olive oil orally before 6 hrs of immobilization stress.

Post-treatments of vitamin

For the post-vitamin stress treatment, the rats were divided into four groups of 10 rats each and were given a single oral dose of vitamin A, E and

C (15 mg/kg of body weight) alone and in combination (vitamin E+C), dissolved in olive oil after 6 hr of stress exposure.

Similar to the pre-vitamin (high dose) stress treatment this set of 10 rats each were given a single dose of fat-soluble vitamin A or E (50 mg/kg body weight) dissolved in olive oil after 6 hr of stress exposure.

3.2.4 Preparation of various extracts

Mako (*Solanum nigrum*)

Fresh leaves of *Solanum nigrum* were collected locally, shade dried and powdered. Aqueous extract was prepared by refluxing with distilled water at 80^0C and concentrated under vacuum. The weight / volume of the extract to solvent after the complete dissolution was fixed at 100 mg/ml for oral administration through catheter.

Garlic (*Allium sativum*)

Sliced and soaked 50 gm of garlic cloves in distilled water overnight at 4^0C. The homogenate was prepared in the mixer and filtered with the help of cheesecloth. The concentration of the filtrate (w/v) was fixed at 100 mg/kg of body weight for oral administration through catheter (Ali and Mohammad, 1986).

Onion (*Allium cepa*)

Fifty gm of onion peel were sliced and soaked overnight at 4^0C in distilled water. The soaked peel were homogenized in a mixer and filtered through cheesecloth. The weight / volume of the extract to solvent after complete dissolution was fixed at 100mg/ml for oral administration.

Extract treatments

The effect of oral administration of various extracts (100 mg/kg body weight) was evaluated on the activities of free radical scavenging enzymes and various other biochemical parameters as mentioned elsewhere in rat

plasma and tissues. The rats were divided into three groups of ten rats each for the extract treatments.

Group 1. To see the effect of the extract of *Solanum nigrum* alone, rats were given a single oral dose of 1ml extract of *Solanum nigrum* (100mg/kg body weight) with the help of catheter.

Group 2. Rats received a single dose of 1ml aqueous garlic extract (100 mg/kg body weight).

Group 3. Rats were given the single dose of onion extract (100 mg/kg body weight) alone to evaluate its pro-oxidant or antioxidant effect on various *in vivo* biochemical parameters involved in scavenging free radicals.

Effect of extract on stress induced changes

In the present study the effect of aqueous extract of *Solanum nigrum* was evaluated on immobilization stress induced changes in rat plasma and tissues, 20 rats were divided into two groups. Rats of one group (10 rats) received the extract (100 mg/kg body weight) orally 1 hr prior to 6 h of immobilization stress exposure (pre-extract stress treated), whereas, the second group (10 rats) received the same dose 1 hr after the immobilization stress session (post-extract stress treated). The controls (n-saline treated) and stress treated rats were run at the same time as discussed earlier.

Effect of garlic and onion extracts on stress induced changes

The effect of aqueous extract of garlic and onion was evaluated on immobilization stress. Similarly as for other extracts and vitamins the animals were divided into four groups. One group (10 rats) received the extract of garlic (100 mg/kg body weight) 1 hr prior to 6 hrs of stress session (pre-garlic extract treated) while the other group (10 rats) received the same dose 1 hr after the stress session (post-garlic extract stress treated). The third group (10 rats) received the extract of onion (100 mg/kg body weight) 1 hr prior to stress (pre-onion extract treated) and the fourth group (10 rats) received the

extract of onion 1 hr after the 6 hr stress session (post-onion extract stress treated).

3.2.5 Catalase (EC 1.11.1.6)

(Beers and Sizer, 1952)

Principle

The enzyme catalase catalyzes the following reaction

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2$$

In the UV range H_2O_2 shows a continual increase in absorption with decreasing wavelength with a maximum at 240 nm. The decomposition of hydrogen peroxide was followed by the loss of absorbance at 240 nm.

Procedure

Three ml of H_2O_2 prepared in 50 mM potassium phosphate buffer pH 7.0, was pipetted into cuvette, 0.025 ml of samples was added as enzyme source, and the contents were mixed thoroughly. The decrease in absorbance at 240 nm was recorded after every 30 seconds for 3 minutes.

Calculation

The specific activity of catalase is defined in terms of micromoles of hydrogen peroxide consumed per minute per milligram of protein sample. The conversion of initial velocity (change in absorbance at 240 nm/min) to catalase specific activity is done as follows.

$$\text{Specific activity (U/mg)} = \frac{\Delta A_{min}^{-1} \times 1000}{43.6 \times \text{mg of protein / ml reaction mixture}}$$

where 43.6 m^{-1} cm^{-1} represents the molar extinction coefficient of hydrogen peroxide.

3.2.6 Superoxide dismutase (EC 1.15.1.1)

(Marklund and Marklund, 1974)

Principle

Procedure depends upon the autoxidation of pyrogallol:

$$\text{Pyrogallol} + O_2 \xrightarrow{\text{autoxidation}} \text{Oxidation products} + O_2$$

$$2O_2 + 2H^+ \longrightarrow O_2 + H_2O_2$$

The increase in absorbance was recorded at 412 nm.

Procedure

Tissues were homogenized (10% w/v) in chilled 0.1 M phosphate buffer (pH 7.0) and centrifuged in cold at 10,000 rpm for 15 minutes. To 0.05 ml of supernatant, 2.85 ml of 0.05 M tris-succinate buffer (pH 8.2) was added, mixed well and incubated at 25^{O}C for 20 minutes. Adding 0.1 ml of 8 nM pyrogallol solution started the reaction. Change in absorbance per minute was immediately recorded for initially 3 minutes at 412 nm on Cintra 5 UV/visible spectrophotometer. A reference set consisting of 0.05 ml distilled water instead of the sample solution, was also run simultaneously.

Calculation

$$\text{SOD Activity} = \frac{(\Delta A/\text{min ref} - \Delta A/\text{min sample}) \times 3}{(\Delta A/\text{min ref}/ 2 \times 0.05 \times \text{units} / 10 \text{ mg tissues})}$$

Where ΔA/min ref = Change of absorbance per min in reference set.

ΔA/min sample = Change of absorbance per minute in sample set.

Activity Unit: One unit of the enzyme is defined as the amount of enzyme, which causes 50% of inhibition of pyrogallol autoxidation under assay conditions.

3.2.7 Glutathione-S-transferase (EC 2.5.1.18)

(Habig *et al*, 1974)

Principle:

The enzyme activity was measured by following the increase in the absorbance at 340 nm of CDNB-GSH conjugate generated as a result of GST

catalysis between glutathione (GSH) and 1-chloro-2,4-dinitro-benzene (CDNB).

$$\text{CDNB} + \text{GSH} \xrightarrow{\text{GST}} \text{CDNB—GSH conjugate}$$

Procedure

Different tissues of rats were homogenized in chilled 0.1 M phosphate buffer pH 7.4 (10% w/v) and centrifuged in cold for 15 min at 15000 rpm. To 0.1 ml of supernatant, 2.7 ml glutathione solution (0.1 M in phosphate buffer, pH 6.5) and 0.2 ml CDNB (1.0 mM in acetone) were added and mixed thoroughly. The change in absorbance at 340 nm was recorded at room temperature against blank containing all the reagents except the enzyme.

Calculation

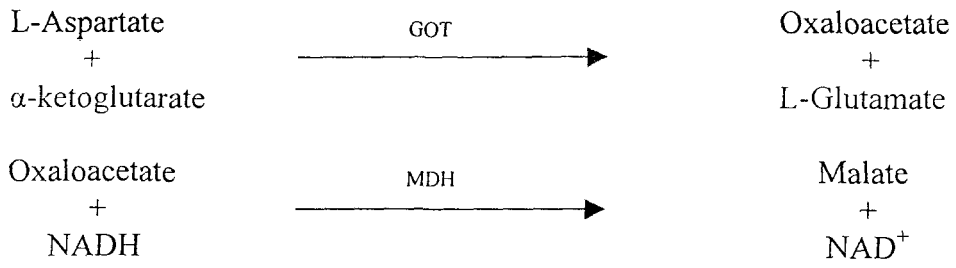
The values were calculated on the basis of molar extinction coefficient of CDNB (9.6 mM^{-1} cm^{-1}) and specific activity of enzyme was expressed in nmoles of GSH-CDNB conjugate formed per minute per mg protein.

3.2.8 Aspartate transaminase (AST or SGOT) (EC 2.6.1.1) (Ladue *et al*, 1954)

The activity of aspartate transaminase was measured using kit from RECKON DIAGNOSTIC PVT. LTD (India).

Principle

The principle was based on the reaction of L-aspartate and α-ketoglutarate in the presence of GOT present in the sample to yield oxaloacetate and L-glutamate. The oxaloacetate thus produced was reduced by malate dehydrogenase (MDH) to yield L-malate with oxidation of NADH to NAD^+. The rate of the reaction was monitored by measurement of the decrease in the absorbance of NADH at 340 nm.



The rate of decrease in absorbance of NADH is proportional to GOT activity in the sample.

Procedure

One ml of working reagent was prepared by mixing enzyme/coenzyme and buffered substrate (90 mM tris-buffer, pH 7.2, 200 mM L-aspartate, 2 mM α-ketoglutarate, 600 U/L MDH and 0.15 mM NADH). To this 0.05 ml of serum was added and mixed. After incubation at $37^{O}C$ for 60 seconds, the absorbance at an interval of 30 seconds was recorded for 2 minutes at 340 nm against distilled water taken as blank.

Calculation

Serum GOT activity (IU/L) = $\Delta A/min \times F$

Where F = 3376 (based on the millimolar extinction coefficient of NADH at 340 nm)

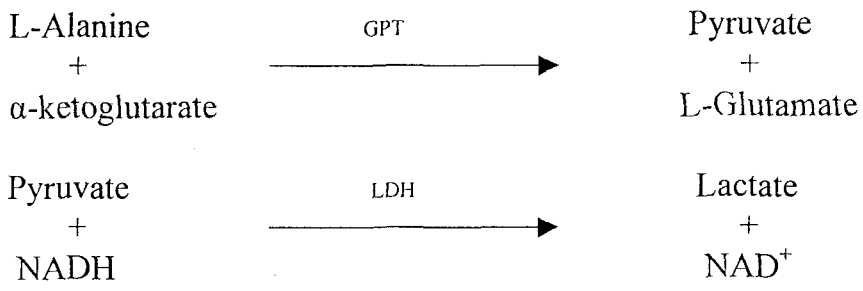
3.2.9 Pyruvate transaminase (ALT or SGPT) (EC 2.6.1.2)

(Henry *et al*, 1960)

This test is done using kit from RECKON DIAGNOSTIC PYT. LTD (India).

Principle

The GPT present in the sample reacted with L-alanine and α-ketoglutarate to yield pyruvate and L-glutamate. Pyruvate thus produced was reduced by lactate dehydrogenase to yield lactate with the oxidation of NADH to NAD^{+}. The reaction was monitored by measurement of the decrease in absorbance of NADH at 340 nm.



The rate of decrease in absorbance was proportional to GPT activity in sample.

Procedure

The procedure was similar to SGOT except the difference was in enzyme/coenzyme and buffered substrate used (90 mM tris-buffer, pH 7.3, 200 mM L-alanine, 2 mM α-ketoglutarate, 2400 U/L LDH and 0.15 mM NADH).

Calculation

Serum GPT activity (IU/L) = ΔA/min x F

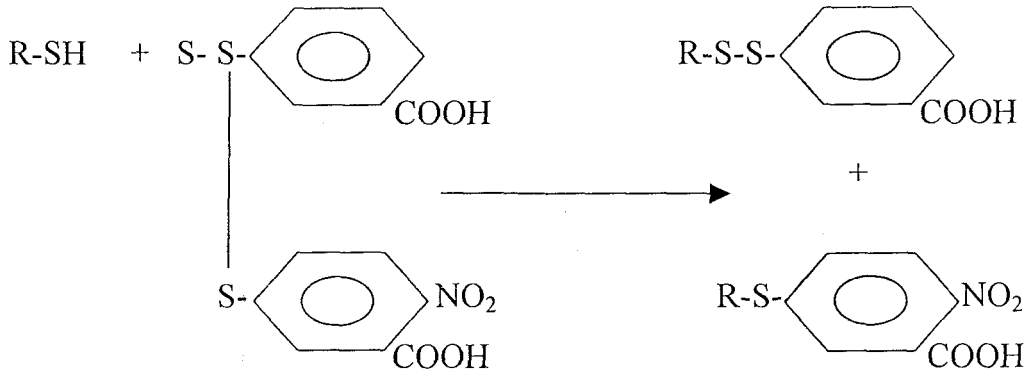
Where F = 3376 (based on the millimolar extinction coefficient of NADH at 340nm).

3.2.10 Sulfhydryl (SH) groups (Sedlak & Lindsay, 1968)

Principle

5-5′ dithiobis-2-nitrobenzoic acid (DTNB) is reduced by SH group of glutathione (GSH) in alkaline medium to produce one mole of 2-nitro-5-mercatpbenzoic acid per mole of –SH group. The reaction is as follows:

The anion (2-nitro-5-mercaptobenzoic acid) has an intense yellow color; it can be used to measure –SH group at 412nm.



2-nitro-5-mercatpbenzoic acid

Procedure

(a) Total SH group

Tissues of rats were homogenized in chilled 0.1 M phosphate buffer pH 7.4 and the volume was adjusted to give 10% (w/v) homogenate. In 0.1 ml of tissue homogenate, 1.5 ml of 0.2 M tris buffer pH 8.2 containing 0.2 M EDTA and 0.1 ml DTNB (0.01M) were added. The mixture was shaken and made to 10 ml with 8.3 ml of absolute methanol. The reaction mixture was centrifuged at 6,000 rpm for 5 minutes in cold. The absorbance of the clear supernatant was read at 412 nm. A calibration curve with different concentration of GSH (200-1600 µmoles) was obtained according to the same procedure as described above. Total SH group in the sample were calculated using the standard curve and the results were expressed as µmoles/g tissue.

(b) Free SH group

One ml tissue (10%) homogenate, prepared in 0.1 M phosphate buffer pH 7.4, was deproteinized by adding 0.1 ml of 10% TCA and centrifuged at 6,000 rpm for 5 minutes. 0.5 ml of aliquot from the clear supernatant was mixed with 0.5 ml distilled water, 0.2 ml of 0.4 M tris buffer in 0.2 M EDTA pH 8.9 and 0.1 ml of 0.01 M DTNB, prepared in absolute methanol, were added to it with proper stirring. The absorbance was read at 412 nm within 30 minutes if the addition of DTNB. A calibration curve with

different concentration of GSH (200-1600 μmoles) was drawn by the same procedure as described above. Free sulfhydryl groups in the sample were calculated using the standard curve and the result were expressed as μmoles/g tissue.

(c) Protein bound SH group

Subtracting free SH from total SH group, as described by Sedlak and Lindsay (1963).

3.2.11 Measurement of Lipid peroxidation

(Ohkawa et al., 1979)

Principle

One molecule of malondialdehyde (MDA) reacted stoichiometrically with two molecules of 2-thiobarbituric acid (TBA) at pH 3.5 according to the following mechanism:

The pink chromogen can be detected stoichiometrically with extinction coefficient of 156 mM^{-1} cm^{-1} at 530 nm.

Procedure

The final volume of the reaction mixture was 4ml, which consisted of 1.5 ml acetic acid (20%), 0.2 ml of SDS (8.1%), 0.7 ml distilled water and 0.1 of tissue homogenate (10% w/v). It was incubated at 95^0C for 60 min, then cooled and centrifuged at 4000 rpm for 10 min. The optical density of MDA formed was read in the supernatant at 532 nm against the blank, treated similarly containing 0.1 ml of distilled water instead of sample.

Calculation

The values were calculated on the basis of molar extinction coefficient of MDA 156 mM^{-1} cm^{-1} and the results were expressed in nmoles of MDA formed per mg protein.

3.2.12 Alkaline phosphatase: (E.C 3.1.3.1)

(Shah et al., 1979)

The activity of alkaline phosphatase in the homogenates and plasma was determined by the method of Shah *et al*, 1979 as modified by Kempson *et al*, 1979. The reaction mixture contained 1.4 ml assay buffer (50 mM glycine, 36 mM NaCl and 45 mM NaOH pH 10.5) and 100 µl sample (10-25 µg plasma or homogenate). The reaction was started by adding 15 µl of 0.6 M p-nitrophenol phosphate (final concentration 6 mM) and incubated at 30^0C for the required time (5-20 min). The reaction was stopped by addition of 50 µl of 5 N NaOH. A calibration curve was prepared simultaneously by using known amount of p-nitrophenol (0.01-0.2 µmoles). The yellow color was read at 405 nm against a reagent blank.

3.2.13 Determination of glucose

(Trinder, 1996)

Principle

For the estimation of glucose, coupled enzymatic method was employed. The aldehyde group of glucose was oxidized by glucose oxidase to give gluconic acid and hydrogen peroxide, which was broken to water and oxygen by peroxidase. The oxygen, thus produced reacted with 4-aminophenazone in the presence of phenol to form a pink colored compound, the intensity of which as determined at 530 nm.

Procedure

Four sets of tubes were taken as unknown (test sample), standard (in duplicate) and blank, 3 ml of glucose reagent (glucose oxidase, peroxidase, 4-aminophenazone, sodium azide and phenol reagent) was added to each test tube, 0.2 ml of serum, 0.02 ml of glucose standard (100mg/dl) and 0.02 ml of distilled water were added to the tubes respectively. The reagent were mixed

and tubes were kept at 37^0C for 15 minutes. The intensities of the color produced were recorded at 530 nm.

Calculation

$$\text{Serum glucose (mg/dl)} = \frac{\text{A unknown}}{\text{A standard}} \times 100$$

Where A = Absorbance at 530 nm.

3.2.14 Uric Acid

(Caraway, 1963)

Principle

Uric acid in the protein free filtrate reacted with phosphotungstic acid reagent in the presence of sodium carbonate (alkaline solution) to form a blue colored complex. The intensity of the color was measured at 710 nm.

Procedure

For the uric acid assay, the deproteinisation of serum was done by taking 1.0 ml of serum, 8.0 ml of distilled water, 0.5 ml sulphuric acid (2/3 N) and 0.5 ml sodium tungstate (10% w/v). After mixing and a wait of 10 minutes, the mixture was centrifuged at 3000 rpm for 10 minutes. 3.0 ml of the supernatant was used for the further assay. To this, 1.0 ml of sodium carbonate (14 % w/v) and 0.1 ml phosphotungstate were added. Standard tube was treated similar to sample containing 3.0 ml of working standard of uric acid (100mg%), after mixing and standing in dark for 15 minutes. Absorbance was measured against the blank treated similar to samples containing 3.0 ml of distilled water that replaces the sample at 710 nm.

Calculation

$$\text{Serum Uric Acid in mg/100 ml} = \frac{\text{O.D test}}{\text{O.D Std}} \times 100$$

3.2.15 Quantitative estimation of protein

(Lowry et al., 1951)

A suitable aliquot of the protein sample was diluted to 1 ml with distilled water. To this 0.5 ml of freshly prepared alkaline copper reagent was added, the alkaline copper reagent was prepared by mixing 0.5% copper Sulphate 1% (w/v), sodium potassium Tartarate 2% (w/v), and sodium carbonate in 0.1 N NaOH in the ratio of 1:1:100, after the incubation for 10 minutes at room temperature, 0.5 ml of 1 N Folin's reagent was added. The contents were rapidly mixed and color intensity was read after 30 minutes against blank at 660 nm. The concentration of protein in the samples was determined using standard curve with BSA.

RESULTS

Controls

Two types of controls were included in the present study, untreated or vehicle treated controls (normal saline / olive oil). A slight difference in the levels of biochemical parameters was observed in these controls.

In the vehicle treated controls (normal saline / olive oil) an insignificant decrease in the activities of free radical scavenging enzymes like SOD, GST and CAT were observed. The level of MDA was found insignificantly enhanced, while the total and free SH group contents were decreased as compared to untreated controls (Table-1).

The serum levels of marker enzymes like ALP, GOT and GPT were found insignificantly enhanced in vehicle treated rats as compared to untreated controls (Table-1).

The pattern of change in the activities of free radical scavenging enzymes and the levels of above mentioned biochemical parameters were found similar in all the tissues like liver, brain, heart, spleen and kidney, and were comparable with their respective circulating levels (Table-2).

The activities of free radical scavenging enzymes like SOD, GST and CAT were found maximum in liver tissues, whereas minimum was found in brain, spleen and heart tissues respectively.

The tissue levels of MDA and total and free SH groups were found maximum in liver tissues. The level of MDA was found minimum in heart, while the tissue levels of total and free SH groups were found least in spleen tissues.

The tissue levels of marker enzymes like ALP, GOT and GPT were found maximum in liver and minimum in brain tissues, while the levels in kidney and spleen were in between (results not shown).

Immobilization Stress

With the increase in the time period of stress from 2 h to 24 h a significant decrease in the circulating activities of free radical scavenging enzymes like SOD ($p<0.001$), GST ($p<0.05$) and catalase ($p<0.001$) was

observed as compared with unstressed controls. However, the plasma levels of MDA ($p<0.05$) showed a significant increase with the duration of stress up to 10 h. (Table- 3).

A slight increase in the activities of free radical metabolizing enzymes and decrease in the levels of MDA was observed at 24 h of restraint stress, but the parameters remained still significantly different from their respective control values (Table-3).

Effect of vitamin treatment

A single oral dose of vitamins was given to rats (15 mg / kg body weight) to see their effects on both the free radical metabolism and various other biochemical markers. The circulating and tissue levels of free radical scavenging enzymes and other biochemical parameters were assayed and compared with their respective vehicle treated controls.

A single oral low dose (15 mg/kg body weight) of vitamin A, E and C either individually or in combination (vitamin E+C) resulted in an insignificant increase in the circulating activities of SOD, GST and catalase. The levels of total and free SH groups were found insignificantly decreased, while the levels of MDA were found insignificantly enhanced as compared to vehicle treated controls (Table- 4).

As compared to vehicle treated controls, the treatment of vitamins resulted in an insignificant decrease in the levels of marker enzymes like serum alkaline phosphatase (SALP), serum glutamate oxaloacetate transaminase (SGOT) and serum glutamate pyruvate transaminase (SGPT) (Table-4).

A single oral high dose (50 mg/kg of body weight) of fat-soluble vitamins A and E was also given to rats to see the comparative effect of a high dose of fat soluble vitamins on free radical metabolizing enzymes and other biochemical parameters.

The oral administration of vitamin A and E (50 mg/kg body weight) resulted in an insignificant increase in the activities of free radical scavenging

enzymes like SOD, GST and catalase, whereas the level of MDA was found insignificantly decreased, and the levels of total and free SH groups were insignificantly enhanced as compared to vehicle treated controls (Table-5).

The levels of marker enzymes like SALP, SGOT and SGPT were also found insignificantly decreased by vitamins A and E treatments (50 mg/kg body weight) when a comparison was made with olive oil treated controls (Table- 5).

Effect of *S. nigrum* extract

The circulating and tissue activities of free radical scavenging enzymes and other biochemical parameters were assayed after the oral treatment of aqueous extract of *S. nigrum* leaves. The treatment resulted in an insignificant increase in the circulating activities of free radical scavenging enzymes like SOD, GST and CAT when compared to normal saline treated controls (Table-6).

The levels of uric acid and MDA were found insignificantly decreased, while the levels of total SH groups and glucose were observed insignificantly increased by the *S. nigrum* leaf extract treatment as compared to vehicle treated controls (Table-6).

The circulating levels of marker enzymes like ALP, GOT and GPT were found insignificantly decreased as compared to vehicle treated controls (Table-6).

Effect of garlic and onion extract

Antioxidant / pro-oxidant effects of both the extracts (garlic and onion) were evaluated in terms of free radical scavenging enzyme activity (SOD, GST, CAT) and various other biochemical parameters (MDA, glucose, uric acid total and free SH groups) and marker enzymes (SALP, SGOT and SGPT). The oral administration of crude extracts of garlic and onion (100 mg/kg of body weight) caused an insignificant increase in the circulating activities of free radical scavenging enzymes like SOD, GST and CAT as

compared to normal saline treated controls. Garlic extract enhanced the levels of these enzymes more than onion, though the increase was not significant (Table-7).

There was an insignificant decrease in the levels of MDA and uric acid by both the extracts treatment, while the levels of total and free SH groups and glucose were found insignificantly enhanced as compared to normal saline treated controls (Table- 7).

The levels of marker enzymes like SALP, SGOT and SGPT were found insignificantly decreased by the extracts treatment when the comparison was made with n-saline treated controls (Table-7).

Effect of antioxidant vitamins (A, E and C) individually and in combination (vitamin E+C) on pre- and post-restraint stress induced oxidative changes

A single oral dose of individual vitamins A, E, C alone and in combination (E+C) 15 mg/kg of body weight was given to rats either prior to (pre-vitamin stress) or after (post-vitamin stress) 6 h of restraint stress treatments.

The treatment of vitamins both before (pre-vitamin stress treatment) and after (post-vitamin stress treatment) stress exposure was found effective in restoring the restraint stress induced alterations in the biochemical parameters towards their control values with a relative dominance by later.

In the pre-vitamin stress treatment studies a significant increase in the circulating activities of SOD ($p<0.001$), GST ($p<0.05$) and catalase ($p<0.05$) (Table-8) was observed as compared to stress alone treated groups. The levels of total and free SH groups ($p<0.05$) were found significantly increased (Table-9), whereas the level of MDA ($p<0.05$) was found significantly decreased as compared to stress treated rats but still remained higher than the control levels (Table-8).

A significant decrease in the levels of marker enzymes like SALP ($p<0.001$), SGOT ($p<0.001$), and SGPT ($p<0.02$) was observed in the pre-vitamin stress treatments as compared to stress treated groups (Table-10).

In the post-vitamin stress treatments, the circulating activities of SOD ($p<0.05$), GST ($p<0.05$) and catalase ($p<0.05$) (Table-8) were found significantly enhanced when a comparison was made with stress alone treated rats. The levels of total and free SH groups ($p<0.05$) were found significantly increased as compared to stress alone treatments (Table-9). A significant reversion towards control values was observed in the antioxidant defense parameters by the post-vitamin stress treatment than pre-vitamin stress treatment (i.e. a resistance to change in the deranged oxidative metabolism by restraint stress).

The elevated levels of marker enzymes due to stress exposure were found significantly decreased (SALP $p<0.05$, SGOT $p<0.05$ and SGPT $p<0.001$) by the post-vitamin treatments as compared to stress alone treated rats (Table-10).

The stress induced altered circulating activities of free radical scavenging enzymes and other biochemical parameters reverted to their respective control values maximally by the post-vitamin stress treatments than pre-vitamin stress treatments, showing a predominant curative than preventive effects of these vitamins.

A single oral high dose (50 mg/kg body weight) of fat-soluble vitamin A and E was also given to the rats to see the effect of high dose of these vitamins on restraint stress induced oxidative changes in rat plasma and tissues, as post-vitamin treatment was found more effective. The high dose of vitamin treatment was found more effective in modulating restraint stress induced biochemical changes in rat plasma and tissues than the low dose of the vitamins i.e.15mg/kg body weight. The circulating activities of SOD ($p<0.001$), GST ($p<0.02$) and catalase ($p<0.02$) in the post-vitamin (50 mg/kg body weight) stress treatment were observed significantly increased as compared to stress treated rats (Table-11). The restraint stress induced

decrease in the levels total and free SH groups were found significantly ($p<0.02$) increased by the post-treatment of vitamin E, whereas the level of MDA ($p<0.02$) was found significantly decreased as compared to stress treated rats (Table-11&12).

The levels of markers enzymes like SALP ($p<0.02$), SGOT ($p<0.001$) and SGPT ($p<0.02$) (Table-13) were found significantly decreased by the post-vitamin (50 mg/kg body weight) stress treatment as compared to their respective controls.

Treatments with both the vitamins A and E (50mg/kg b.w.) was found effective in restoring the stress induced changes towards their control values in circulating and tissue levels of rats. However, the treatment with vitamin E was found more effective than vitamin A. When compared among the tissues the maximum antioxidant effect of these vitamins was observed in liver and minimum in brain. Though the effect of these vitamins on brain tissue levels of these antioxidative parameters was least, but significant.

In liver tissue, the activities of SOD ($p<0.02$), GST ($p<0.02$) and catalase ($p<0.001$) (Table-14) were significantly enhanced in both post and pre-vitamin stress treatment as compared to stress alone treatments. The stress induced decrease in the tissue levels of total and free SH groups were found increased significantly ($p<0.02$) by both pre and post-vitamin stress treatments, while the effects of later was found more pronounced (Table-15).

The enhanced liver levels of marker enzymes like ALP ($p<0.02$), GOT ($p<0.02$) and GPT ($p<0.02$) by the stress treatment were found significantly decreased by the vitamin treatments (Table-16), but still remained significantly enhanced than their respective control values. Here too the post-vitamin stress treatment was found more effective than pre-vitamin stress treatment, though a marked reversion towards control values was observed.

The post-vitamin stress treatment resulted in a significant increase in the brain activities of SOD ($p<0.001$), GST ($p<0.02$) and catalase ($p<0.05$) as compared to stress alone treated rats (Table-14).

The levels of total and free SH groups ($p<0.02$) (Table-15) in the brain tissue were found significantly enhanced, while the level of MDA ($p<0.05$) (Table-14) was observed significantly decreased by the vitamin treatments as compared to stress treated rats, but when compared to controls the values were not much different.

An inter-tissue comparison of vitamin treatments revealed a maximum effect both curative and preventive in liver while minimum but significant effect was observed in brain tissues too.

The pattern of change in the above mentioned parameters by vitamin treatments was observed nearly the same in the tissues like liver, brain, heart, spleen and kidney, though the extent of variation was different which can be summarized from maximum to minimum as follows:

Liver > Kidney > Heart > Spleen > Brain

The effectiveness of treatments of vitamins individually and in combination can be summarized as follows:

Vitamin E + C ≥ Vitamin E > Vitamin C > Vitamin A

Effect of aqueous extract of *S. nigrum* on restraint stress induced changes in rat plasma and tissues

A single oral dose of aqueous extract of *S. nigrum* leaves (100 mg/kg of body weight) was given to rats both before (pre-extract stress treatment) and after (post-extract stress treatment) 6 h of restraint stress exposure, to evaluate the possible preventive (resistance to change in oxidative metabolism towards stress) and curative (reversion towards control values after stress) effects on restraint stress induced biochemical changes in rat plasma and tissues.

By the treatment of extract prior to restraint stress, the circulating activities of SOD, GST and catalase were found significantly increased ($p<0.05$, $p<0.001$, $p<0.001$ respectively) as compared to stress alone treatment but still remained significantly lowered than controls (Fig-1. a, b,

Table-1. Effect of normal saline and olive oil on circulating levels of SOD, GST, catalase, MDA, Total and Free SH groups, ALP, SGOT and SGPT.

	SOD	Catalase	GST	MDA	Total SH	Free SH	ALP	SGOT	SGPT
Control (Untreated)	5.441 ± 0.351	0.602 ± 0.114	0.509 ± 0.017	0.642 ± 0.035	0.612 ± 0.021	0.094 ± 0.001	0.071 ± 0.001	51.23 ± 2.365	35.22 ± 2.012
n-saline	5.011 ± 0.214	0.541 ± 0.112	0.401 ± 0.101	0.761 ± 0.024	0.512 ± 0.012	0.071 ± 0.002	0.089 ± 0.002	59.214 ± 2.045	42.235 ± 3.021
Olive Oil	5.213 ± 0.521	0.551 ± 0.125	0.452 ± 0.012	0.681 ± 0.014	0.561 ± 0.021	0.082 ± 0.012	0.081 ± 0.003	55.026 ± 3.015	38.325 ± 4.021

Table-2. Effect of normal saline and olive oil on tissues levels of SOD, GST, catalase, MDA, SH groups.

Tissues		SOD *Units/mg protein*	GST nmoles/mg protein	CAT Units/mg protein	MDA nmoles/mg of protein	SH Groups μmoles/g tissue
Liver	Control	16.023 ± 2.032	142.32 ± 5.036	8.021 ± 1.64	7.021 ± 1.640	11.021 ± 0.321
	n- saline	14.021 ± 1.542	135.25 ± 6.485	7.021 ± 1.12	8.021 ± 0.214	10.210 ± 0.153
	Olive Oil	12.021 ± 1.856	121.021 ± 4.052	6.021 ± 0.954	8.932 ± 0.346	9.021 ± 1.024
Heart	Control	9.032 ± 0.956	115.021 ± 5.498	1.921 ± 0.095	5.032 ± 2.011	3.025 ± 0.154
	n-saline	8.321 ± 2.654	112.021 ± 3.483	1.832 ± 0.014	6.532 ± 1.641	2.653 ± 0.214
	Olive Oil	7.132 ± 1.11	102.256 ± 7.049	1.230 ± 0.023	7.210 ± 2.001	1.932 ± 0.469
Brain	Control	5.021 ± 0.864	96.451 ± 2.421	5.021 ± 0.153	9.021 ± 2.140	10.65 ± 1.513
	n-saline	4.321 ± 0.051	86.051 ± 7.046	4.653 ± 0.134	10.84 ± 1.054	9.547 ± 0.214
	Olive Oil	3.021 ± 1.021	79.021 ± 4.001	3.678 ± 0.146	11.488 ± 0.153	9.018 ± 1.246

Table-3. Effect of different time periods of Immobilization stress induced changes in circulating levels of SOD, Catalase, GST and MDA.

Time period of stress	SOD Unit/mg protein	Catalase Unit/mg protein	GST nmoles/mg protein	MDA nmoles/mg protein
Control (10)	26.4 ± 2.88	0.037 ± 0.001	1.02 ± 0.19	3.14 ± 0.47
2 h Stress (10)	23.1^{a} ± 3.34	0.031^{b} ± 0.003	0.047^{c} ± 0.06	4.53^{a} ± 0.52
4 h Stress (10)	15.76^{a} ± 1.87	0.024^{b} ± 0.001	0.30^{c} ± 0.03	5.07^{c} ± 0.44
6 h Stress (10)	8.00^{c} ± 0.61	0.016^{c} ± 0.02	0.27^{c} ± 0.06	6.84^{a} ± 0.57
10 h Stress (10)	7.04^{c} ± 1.10	0.012^{a} ± 0.003	0.25^{c} ± 0.06	9.53^{c} ± 1.76
12 h Stress (10)	12.25^{c} ± 2.99	0.015^{c} ± 0.001	0.31^{b} ± 0.05	8.92^{b} ± 2.39
24 h Stress (10)	14.17^{b} ± 2.90	0.017^{a} ± 0.002	0.39^{b} ± 0.05	8.03^{a} ± 1.90

Number of experimental rats is indicated in the parenthesis. a = $p<0.02$, b = $p<0.05$, c = $p<0.001$ as compared with controls

Table-4. Effect of antioxidant vitamin A, E and C individually and in combination (E+C), (low dose) on circulating levels of SOD, GST, catalase, MDA, Total and Free SH groups, ALP, SGOT and SGPT.

	SOD	Catalase	GST	MDA	Total SH	Free SH	ALP	SGOT	SGPT
Control (olive oil)	5.44 ± 0.351	0.60 ± 0.114	0.50 ± 0.017	0.64 ± 0.035	0.612 ± 0.021	0.094 ± 0.001	0.071 ± 0.001	28.36 ± 0.791	18.25 ± 1.215
Vit A	5.69 ± 0.265	0.69 ± 0.01	0.59 ± 0.04	0.57 ± 0.06	0.721 ± 0.012	0.119 ± 0.005	0.064 ± 0.003	26.325 ± 2.154	16.251 ± 2.012
Vit E	6.21 ± 1.012	0.71 ± 0.003	0.62 ±0.02	0.51 ± 0.01	0.861 ± 0.03	0.102 ± 0.01	0.051 ± 0.004	24.151 ± 2.514	17.21 ± 0.214
Vit C	5.61 ± 0.214	0.65 ± 0.021	0.70 ± 0.01	0.62 ± 0.120	0.652 ± 0.210	0.107 ± 0.012	0.059 ± 0.001	25.41 ± 2.012	18.21 ± 2.514
Vit (E+C)	6.01 ± 0.012	0.70 ± 0.001	0.71 ± 0.02	0.42 ± 0.031	0.91 ± 0.021	0.099 ± 0.004	0.043 ± 0.052	22.03 ± 3.021	16.01 ± 4.021

Table-5. Effect of antioxidant vitamin A, E and C individually and in combination (E+C), (low dose) on circulating levels of SOD, GST, catalase, MDA, Total and Free SH groups, ALP, SGOT and SGPT.

	SOD	Catalase	GST	MDA	Total SH	Free SH	ALP	SGOT	SGPT
Control (Olive oil)	8.739 ± 0.193	0.532 ± 0.056	0.184 ± 0.002	1.288 ± 0.021	0.599 ± 0.148	0.152 ± 0.001	0.067 ± 0.002	35.251 ± 3.215	21.325 ± 3.215
Vit A	9.012 ± 0.213	0.632 ± 0.156	0.201 ± 0.123	1.215 ± 0.231	0.612 ± 0.021	0.165 ± 0.002	0.059 ± 0.013	27.215 ± 2.145	19.215 ± 2.012
Vit E	10.213 ± 0.112	0.691 ± 0.315	0.313 ± 0.213	1.012 ± 0.089	0.721 ± 0.091	0.197 ± 0.003	0.701 ± 0.124	26.215 ± 1.201	19.011 ± 2.015

Table-6. Effect of normal saline and *S. nigrum* leaves extract on circulating levels of SOD, GST, catalase, MDA, Total SH groups, ALP, SGOT, SGPT, glucose and uric acid.

	SOD	Catalase	GST	MDA	Total SH	ALP	SGOT	SGPT	Glucose	Uric Acid
Control	2.66 ± 0.63	0.75 ± 0.06	0.25 ± 0.03	5.65 ± 0.36	0.75 ± 0.06	0.04 ± 0.01	33.27 ± 0.62	7.912 ± 0.13	174.5 ± 19.97	7.01 ± 1.55
n-saline	2.32 ± 0.025	0.61 ± 0.01	0.19 ± 0.021	5.92 ± 0.01	0.61 ± 0.01	0.051 ± 0.001	34.20 ± 2.01	8.212 ± 2.01	161.01 ± 6.021	8.021 ± 1.521
Mako	2.92 ± 0.15	0.76 ± 0.02	0.32 ± 0.03	4.86 ± 0.28	0.92 ± 0.04	0.03 ± 0.001	29.33 ± 1.24	6s.13 ± 0.22	185.91 ± 6.04	5.60 ± 0.59

Table-7. Effect of normal saline, extract of garlic and onion on circulating levels of SOD, GST, catalase, MDA, Total and Free SH groups, ALP, SGOT, SGPT, glucose and uric acid.

	SOD	Catalase	GST	MDA	Total SH	Free SH	ALP	SGOT	SGPT	Glucose	Uric Acid
Control (n-saline)	4.90 ± 0.11	0.95 ± 0.03	0.82 ± 0.02	6.02 ± 0.13	0.96 ± 0.05	0.13 ± 0.001	0.13 ± 0.01	94.63 ± 5.62	39.48 ± 1.52	162.03 ± 6.02	8.27 ± 0.15
n-saline	3.92 ± 0.124	0.81 ± 0.01	0.72 ± 0.05	7.02 ± 1.02	0.82 ± 0.02	0.20 ± 0.02	0.21 ± 0.01	101.21 ± 4.521	42.30 ± 2.01	158.21 ± 3.024	7.62 ± 2.01
Onion	5.01 ± 0.32	1.02 ± 0.06	1.13 ± 0.02	5.02 ± 0.45	1.13 ± 0.05	0.14 ± 0.001	0.10 ± 0.01	92.65 ± 5.38	36.63 ± 2.67	163.02 ± 6.02	8.45 ± 1.52
Garlic	5.13 ± 0.06	1.16 ± 0.01	1.13 ± 0.08	4.87 ± 0.15	1.06 ± 0.09	0.17 ± 0.003	0.12 ± 0.01	86.97 ± 3.75	33.52 ± 1.97	171.27 ±5.33	7.65 ± 0.25

Table-8. **Effect of treatment of Vitamin A, E and C individually and in combination (vitamin E + C) on Immobilization stress induced changes in circulating levels of SOD, Catalase, GST and MDA.**

		SOD Unit/mg protein	Catalase Unit/mg protein	GST nmoles/mg protein	MDA nmoles/mg protein
Controls (10)		5.44 ± 0.351	0.60 ± 0.014	0.50 ± 0.017	0.64 ± 0.035
Stressed (10)		3.22^{c} ± 0.291	0.40^{c} ± 0.02	0.23^{b} ± 0.017	0.97^{a} ± 0.043
Pre-vitamin stressed treated (10) each	Vit A	$3.45^{aa'}$ ± 0.21	0.47^{a} ± 0.022	$0.30^{ba'}$ ± 0.034	0.76^{b} ± 0.024
	Vit E	$3.8^{bc'}$ ± 0.070	$0.52^{ab'}$ ± 0.016	$0.42^{cb'}$ ± 0.037	$0.77^{cb'}$ ± 0.030
	Vit C	$3.55^{aa'}$ ± 0.153	$0.44^{ba'}$ ± 0.020	$0.29^{ca'}$ ± 0.047	$0.72^{aa'}$ ± 0.031
	Vit (E+C)	$3.86^{ba'}$ ± 0.129	$0.53^{aa'}$ ± 0.011	$0.49^{ac'}$ ±0.024	0.68^{a} ± 0.022
Post-vitamin stressed treated (10) each	Vit A	3.50^{c} ± 0.116	0.55^{c} ± 0.011	$0.36^{ca'}$ ±0.030	0.68^{a} ± 0.035
	Vit E	$4.2^{cb'}$ ± 0.24	$0.53^{cb'}$ ± 0.013	$0.43^{ba'}$ ±0.036	$0.53^{aa'}$ ± 0.028
	Vit C	$4.01^{ca'}$ ± 0.20	$0.49^{ca'}$ ± 0.015	0.32^{b} ±0.025	$0.65^{ac'}$ ± 0.02
	Vit (E+C)	4.42^{a} ± 0.125	$0.53^{ca'}$ ± 0.016	$0.40^{bb'}$ ±0.033	0.63^{a} ± 0.022

Number of experimental rats is indicated in the parenthesis. a = $p<0.02$, b = $p<0.05$, c = $p<0.001$ as compared with controls, where as a' = $p<0.02$, b' = $p<0.05$ and c' = $p<0.001$ as compared to stressed rats.

Table-9. **Effect of treatment of Vitamin A, E and C individually and in combination (vitamin E + C) on Immobilization stress induced changes in circulating levels of total and free SH groups.**

		GSH µmoles/g tissue	Free GSH µmoles/g tissue
Controls (10)		0.612 ± 0.021	0.094 ± 0.001
Stressed (10)		0.32^{c} ± 0.015	0.042^{c} ± 0.004
Pre-vitamin stressed treated (10) each	Vit A	$0.401^{aa'}$ ± 0.014	0.051^{c} ± 0.004
	Vit E	$0.561^{bb'}$ ± 0.031	$0.072^{ac'}$ ± 0.001
	Vit C	0.49^{b} ± 0.001	$0.062^{ba'}$ ± 0.005
	Vit (E+C)	$0.560^{cc'}$ ± 0.009	$0.071^{ba'}$ ± 0.004
Post-vitamin stressed treated (10) each	Vit A	$0.456^{aa'}$ ± 0.014	0.061^{b} ± 0.001
	Vit E	$0.601^{cb'}$ ± 0.042	$0.091^{ca'}$ ± 0.01
	Vit C	0.591^{a} ± 0.021	0.070^{b} ± 0.012
	Vit (E+C)	$0.601^{ba'}$ ± 0.031	$0.089^{ca'}$ ± 0.014

Number of experimental rats is indicated in the parenthesis. a = $p<0.02$, b = $p<0.05$, c = $p<0.001$ as compared with controls, where as a' = $p<0.02$, b' = $p<0.05$ and c' = $p<0.001$ as compared to stressed rats.

Table-10. **Effect of treatment of Vitamin A, E and C individually and in combination (vitamin E + C) on Immobilization stress induced changes in circulating levels of ALP, SGOT and SGPT.**

		ALP nmol/min/ mg of protein	SGOT Units	SGPT Units
Controls (10)		0.071 ± 0.001	28.36 ± 0.791	18.25 ± 1.215
Stressed (10)		0.138^{c} ± 0.01	41.02^{b} ± 2.041	26.02^{a} ± 3.021
Pre-vitamin stressed treated (10) each	Vit A	$1.211^{ac'}$ ± 0.05	$37.31^{bc'}$ ± 3.11	$24.14^{aa'}$ ± 1.54
	Vit E	$0.901^{ca'}$ ± 0.04	$33.65^{ba'}$ ± 3.54	$21.51^{ab'}$ ± 2.15
	Vit C	1.150^{b} ± 0.01	35.24^{c} ± 2.35	23.56^{b} ± 2.65
	Vit (E+C)	$0.812^{ac'}$ ± 0.02	$32.12^{ba'}$ ± 3.21	$20.01^{ab'}$ ± 1.21
Post-vitamin stressed treated (10) each	Vit A	$1.120^{ac'}$ ± 0.03	$36.51^{cb'}$ ± 3.40	$24.61^{cc'}$ ± 0.95
	Vit E	$1.02^{ba'}$ ± 0.02	$32.54^{bb'}$ ± 2.51	21.51^{b} ± 2.54
	Vit C	$1.05^{ab'}$ ± 0.05	$35.62^{ba'}$ ± 2.45	$23.54^{ba'}$ ± 1.01
	Vit (E+C)	$0.075^{aa'}$ ± 0.001	$30.21^{aa'}$ ± 2.35	$19.51^{bb'}$ ± 1.54

Number of experimental rats is indicated in the parenthesis. a = $p<0.02$, b = $p<0.05$, c = $p<0.001$ as compared with controls, where as a' = $p<0.02$, b' = $p<0.05$ and c' = $p<0.001$ as compared to stressed rats.

Table-11. **Effect of high dose of vitamin A and E on Immobilization stress induced changes in circulating levels of SOD, catalase, GST and MDA.**

	SOD Unit/mg protein	Catalase Unit/mg protein	GST nmoles/mg protein	MDA nmoles/mg of protein
Control (10)	8.739 ± 0.193	0.532 ± 0.065	0.184 ± 0.002	1.288 ± 0.021
Stressed (10)	2.921^{c} ± 0.110	0.288^{b} ± 0.062	0.090^{c} ± 0.041	3.194^{c} ± 0.132
Vitamin A (10)	9.012 ± 0.213	0.632 ± 0.156	0.201 ± 0.123	1.215 ± 0.231
Vitamin E (10)	10.213 ± 0.112	0.691 ± 0.315	0.313 ± 0.213	1.012 ± 0.089
Post-vitamin A stressed treated (10)	$3.367^{ba'}$ ± 0.729	$0.385^{bc'}$ ± 0.065	$0.132^{ab'}$ ± 0.018	$2.375^{bc'}$ ± 0.139
Post-vitamin E stressed treated (10)	$8.777^{ac'}$ ± 0.387	$0.483^{aa'}$ ± 0.049	$0.147^{ca'}$ ± 0.008	$1.227^{ca'}$ ± 0.152

Number of experimental rats is indicated in the parenthesis. a = $p<0.02$, b = $p<0.05$, c = $p<0.001$ as compared with controls, where as a' = $p<0.02$, b' = $p<0.05$ and c' = $p<0.001$ as compared to stressed rats.

Table-12. **Effect of high dose of vitamin A and E on Immobilization stress induced changes in circulating levels of total and free SH groups.**

	GSH µmoles/g tissue	Free GSH µmoles/g tissue
Control (10)	0.599 ± 0.148	0.152 ± 0.001
Stressed (10)	0.396^{b} ± 0.052	0.056^{b} ± 0.001
Vitamin A (10)	0.612 ± 1.021	0.165 ± 0.002
Vitamin E (10)	0.721 ± 0.912	0.197 ± 0.003
Post-vitamin A stressed treated (10)	$0.524^{aa'}$ ± 0.089	$0.172^{ac'}$ ± 0.042
Post-vitamin E stressed treated (10)	$0.602^{ba'}$ ± 0.097	$0.201^{ca'}$ ± 0.001

Number of experimental rats is indicated in the parenthesis. a = $p<0.02$, b = $p<0.05$, c = $p<0.001$ as compared with controls, where as a' = $p<0.02$, b' = $p<0.05$ and c' = $p<0.001$ as compared to stressed rats.

Table-13. **Effect of high dose of vitamin A and E on Immobilization stress induced changes in circulating levels of GOT, GPT and ALP.**

	SGOT Units	SGPT Units	ALP nmol/min/mg of protein
Control (10)	54.214 ± 2.021	35.321 ± 2.352	0.067 ± 0.012
Stressed (10)	65.321^{c} ± 5.365	49.325^{b} ± 4.251	0.089^{a} ± 0.009
Vitamin A (10)	64.214 ± 4.350	44.521 ± 6.021	0.059 ± 0.132
Vitamin E (10)	63.232 ± 6.021	43.021 ± 5.021	0.701 ± 0.214
Post-vitamin A stressed treated (10)	$60.321^{ab'}$ ± 5.045	$42.321^{ba'}$ ± 2.035	$0.071^{ba'}$ ± 0.005
Post-vitamin E stressed treated (10)	$57.201^{ac'}$ ± 5.985	$36.214^{ca'}$ ± 4.654	0.061^{ba} ± 0.006

Number of experimental rats is indicated in the parenthesis. a = $p<0.02$, b = $p<0.05$, c = $p<0.001$ as compared with controls, where as a' = $p<0.02$, b' = $p<0.05$ and c' = $p<0.001$ as compared to stressed rats.

Table-14. **Effect of treatment of Vitamin A, E and C individually and in combination (vitamin E+C) on Immobilization stress induced changes in Liver tissue levels of SOD, GST, Catalase and MDA.**

		SOD Unit/mg protein	Catalase Unit/mg protein	GST nmoles/mg protein	MDA nmoles/mg of protein
Controls (10)		18.340 ± 0.551	10.355 ± 0.354	174.391 ± 3.723	8.394 ± 0.665
Stressed (10)		10.060^{c} ± 0.903	4.353^{c} ± 0.359	121.166^{c} ± 4.708	17.236^{c} ± 0.721
Pre-vitamin stress treated (10) each	Vit A	$12.301^{ac'}$ ± 1.331	$6.018^{ba'}$ ± 0.397	$134.235^{ac'}$ ± 4.979	$16.627^{ba'}$ ± 0.577
	Vit E	$12.995^{ba'}$ ± 1.275	$7.551^{cc'}$ ± 0.596	$157.346^{cc'}$ ± 4.917	$12.326^{cc'}$ ± 0.449
	Vit C	12.705^{b} ± 1.176	$6.915^{cc'}$ ± 0.791	$149.366^{ab'}$ ± 5.349	14.323^{b} ± 0.801
	Vit (E+C)	$13.165^{ba'}$ ± 1.186	$7.706^{ba'}$ ± 1.323	155.326^{c} ± 4.661	$13.124^{ca'}$ ± 0.606
Post-vitamin stress treated (10) each	Vit A	$13.596^{aa'}$ ± 0.615	7.694^{b} ± 1.358	$152.333^{ca'}$ ± 4.873	15.320^{c} ± 0.497
	Vit E	$16.934^{ca'}$ ± 0.586	$9.366^{cc'}$ ± 1.396	$164.257^{ba'}$ ± 5.184	$9.322^{bb'}$ ± 0.442
	Vit C	16.015^{a} ± 0.687	8.303^{a} ± 0.954	155.628^{c} ± 4.748	$10.234^{bb'}$ ± 0.428
	Vit (E+C)	$17.014^{cb'}$ ± 0.489	$9.169^{ab'}$ ± 1.359	$165.327^{ab'}$ ± 5.167	$9.011^{ca'}$ ± 0.556

Number of experimental rats is indicated in the parenthesis. a = $p<0.02$, b = $p<0.05$, c = $p<0.001$ as compared with controls, where as a' = $p<0.02$, b' = $p<0.05$ and c' = $p<0.001$ as compared to stressed rats.

Table-15. **Effect of treatment of Vitamin A, E and C individually and in combination (vitamin E+C) on Immobilization stress induced changes in Liver tissue levels of total and free SH groups.**

		GSH µmoles/g tissue	Free GSH µmoles/g tissue
Controls (10)		15.014 ± 1.023	2.316 ± 0.322
Stressed (10)		6.132^{c} ± 0.965	0.965^{c} ± 0.212
Pre-vitamin stressed treated (10) each	Vit A	$7.015^{ac'}$ ± 1.231	1.212^{b} ± 0.314
	Vit E	$10.036^{cc'}$ ± 0.932	$1.899^{cc'}$ ± 0.351
	Vit C	8.304^{c} ± 1.641	0.217^{c} ± 0.13
	Vit (E+C)	$12.016^{aa'}$ ± 0.321	$1.017^{bb'}$ ± 0.011
Post-vitamin stressed treated (10) each	Vit A	$8.324^{bc'}$ ± 0.354	1.036^{b} ± 0.021
	Vit E	$13.369^{ba'}$ ± 1.035	$2.012^{ba'}$ ± 0.215
	Vit C	$11.927^{ba'}$ ± 0.31	1.351^{a} ± 0.381
	Vit (E+C)	$14.038^{ac'}$ ± 0.329	$2.214^{ab'}$ ±1.021

Number of experimental rats is indicated in the parenthesis. a = $p<0.02$, b = $p<0.05$, c = $p<0.001$ as compared with controls, where as a' = $p<0.02$, b' = $p<0.05$ and c' = $p<0.001$ as compared to stressed rats.

Table-16. **Effect of treatment of Vitamin A, E and C individually and in combination (vitamin E+C) on Immobilization stress induced changes in Liver tissue levels of GOT, GPT and ALP.**

		GOT Units	GPT Units	ALP nmol/min/mg of protein
Controls (10)		7.124 ± 1.012	4.456 ± 5.015	0.832 ± 0.06
Stressed (10)		13.145^{b} ± 2.012	7.453^{b} ± 2.014	1.731^{c} ± 0.001
Pre-vitamin stressed treated (10) each	Vit A	11.235^{a} ± 2.012	$7.632^{aa'}$ ± 0.02	$0.632^{cb'}$ ± 2.953
	Vit E	$9.014^{ac'}$ ±1.014	$6.510^{cb'}$ ± 0.03	$0.532^{ba'}$ ± 4.755
	Vit C	10.04^{b} ± 3.156	$7.215^{cb'}$ ± 0.051	0.604^{b} ± 1.235
	Vit (E+C)	$8.589^{ac'}$ ± 1.320	6.465^{c} ± 0.003	0.447^{a} ± 3.265
Post-vitamin stressed treated (10) each	Vit A	$12..012^{ca'}$ ± 1.596	$5.354^{ca'}$ ± 0.065	$0.713^{ba'}$ ± 4.135
	Vit E	$8.235^{aa'}$ ± 5.124	$4.162^{ba'}$ ± 0.065	$0.562^{ab'}$ ± 4.265
	Vit C	9.001^{c} ± 2.356	4.932^{a} ± 0.054	0.621^{a} ± 3.247
	Vit (E+C)	$8.01^{ba'}$ ± 4.568	$4.124^{ca'}$ ± 0.086	0.536^{b} ±5.132

Number of experimental rats is indicated in the parenthesis. a = $p<0.02$, b = $p<0.05$, c = $p<0.001$ as compared with controls, where as a' = $p<0.02$, b' = $p<0.05$ and c' = $p<0.001$ as compared to stressed rats.

Table-17. **Effect of treatment of Vitamin A, E and C individually and in combination (vitamin E+C) on Immobilization stress induced changes in Brain tissue levels of SOD, Catalase, GST and MDA.**

		SOD Unit/mg protein	Catalase Unit/mg protein	GST nmoles/mg protein	MDA nmoles/mg of protein
Controls (10)		3.35 ±.0.04	7.38 ±.0.35	141.23 ±.9.03	7.93 ±0.60
Stressed rats (10)		1.98^{c} ± 0.04	$5.39^{c'}$ ±0.63	$103.53^{c'}$ ±0.017	$11.37^{c'}$ ±0.62
Pre-vitamin stress treated (10) each	Vit A	$2.10^{ab'}$ ± 0.13	$5.50^{ba'}$ ± 0.65	$112.65^{ab'}$ ± 3.44	$11.01^{aa'}$ ± 0.34
	Vit E	$2.30^{ba'}$ ± 0.13	$5.90^{aa'}$ ± 0.32	$119.39^{ca'}$ ± 1.07	10.35^{b} ± 0.21
	Vit C	2.20^{a} ± 0.13	$5.85^{ba'}$ ± 0.95	118.89^{a} ± 1.14	$10.64^{ab'}$ ± 0.88
	Vit (E+C)	$2.33^{ac'}$ ± 0.12	5.96^{a} ± 0.56	$120.58^{ba'}$ ± 1.74	10.32^{a} ± 0.53
Post-vitamin stressed treated (10) each	Vit A	2.21^{a} ± 0.13	$5.70^{ac'}$ ± 0.94	118.69^{c} ± 1.21	$10.34^{bc'}$ ± 0.39
	Vit E	$2.28^{ac'}$ ± 0.13	$6.30^{ab'}$ ± 0.89	$129.96^{aa'}$ ± 2.11	9.63^{b} ± 0.26
	Vit C	2.25^{b} ± 0.14	$6.10^{ca'}$ ± 0.72	124.34^{b} ± 1.88	$10.13^{ca'}$ ± 0.60
	Vit (E+C)	$2.30^{ba'}$ ± 0.17	$6.31^{ca'}$ ± 0.79	$130.01^{cb'}$ ± 3.22	$10.10^{ca'}$ ± 0.75

Number of experimental rats is indicated in the parenthesis. a = $p<0.02$, b = $p<0.05$, c = $p<0.001$ as compared with controls, where as a' = $p<0.02$, b' = $p<0.05$ and c' = $p<0.001$ as compared to stressed rats.

Table-18. Effect of treatment of Vitamin A, E and C individually and in combination (vitamin E+C) on Immobilization stress induced changes in Brain tissue levels of total, free SH groups and ALP..

		GSH μmoles/g tissue	Free GSH μmoles/g tissue	ALP nmol/min/mg of protein
Controls (10)		11.32 ± 0.985	1.612 ±0.021	0.658 ± 0.054
Stressed (10)		5.84[c] ±0.678	0.042[b] ± 0.032	1.532[a] ±0.021
Pre-vitamin stress treated (10) each	Vit A	6.13[a b'] ± 0.496	0.068[a a'] ±0.123	1.365[b a'] ±0.214
	Vit E	7.01[b a'] ±0.671	1.063[b b'] ±0.321	1.025[b] ±0.079
	Vit C	6.64[a] ± 1.09	0.091[a] ±0.152	1.102[a] ±0.054
	Vit (E+C)	7.10[b a'] ±0.865	1.103[b a'] ± 0.254	0.921[b a'] ±0.021
Post-vitamin stress treated (10) each	Vit A	6.89[a] ±1.035	0.954[a] ± 0.065	1.199[b a'] ±0.085
	Vit E	9.93[a a'] ±1.351	1.542[c a'] ± 0.652	0.796[c a'] ±0.015
	Vit C	7.86[a] ± 0.489	1.210[a] ± 0.549	0.932[a] ± 0.084
	Vit (E+C)	10.12[c a'] ± 1.23	1.542[b a'] ± 0.354	0.536[c b'] ± 0.076

Number of experimental rats is indicated in the parenthesis, a = p<0.02, b = p<0.05, c = p<0.001 as compared with controls, where as a' = p<0.02, b' = p<0.05 and c' = p<0.001 as compared to stressed rats.

Table-19. **Effect of treatment of Vitamin A, E and C individually and in combination (vitamin E + C) on Immobilization stress induced changes in Heart tissue levels of SOD, Catalase, GST and MDA.**

		SOD Unit/mg protein	Catalase Unit/mg protein	GST nmoles/mg protein	MDA nmoles/mg protein
Controls (10)		8.135 ± 0.012	1.014 ± 0.124	135.178 ± 1.324	6.014 ± 0.642
Stressed (10)		3.156^{b} ± 0.124	0.412^{c} ± 0.045	62.456^{c} ± 1.456	18.156^{b} ± 0.541
Pre-vitamin stressed treated (10) each	Vit A	$4.015^{ab'}$ ± 1.235	$0.669^{bb'}$ ± 0.345	$89.156^{ca'}$ ± 2.014	$16.012^{cb'}$ ± 0.145
	Vit E	$5.014^{ba'}$ ± 1.458	$0.812^{ba'}$ ± 0.146	$108.345^{ba'}$ ± 1.359	$11.124^{ca'}$ ± 0.314
	Vit C	4.412^{b} ± 0.955	$0.712^{cb'}$ ± 0.013	$95.065^{ac'}$ ± 3.015	$13.014^{bb'}$ ± 0.245
	Vit (E+C)	$5.902^{bc'}$ ± 1.420	$0.929^{aa'}$ ± 0.019	$117.015^{ba'}$ ± 2.488	$10.015^{ca'}$ ± 0.146
Post-vitamin stressed treated (10) each	Vit A	$6.014^{ba'}$ ± 1.234	0.821^{c} ± 0.164	$99.012^{ca'}$ ± 2.654	$13.123^{cb'}$ ± 0.512
	Vit E	$8.210^{c'}$ ± 0.924	$1.913^{ab'}$ ± 0.431	$128.235^{cb'}$ ± 2.145	$7.014^{aa'}$ ± 0.481
	Vit C	$7.990^{aa'}$ ± 0.145	$0.913^{ac'}$ ± 0.001	$108.145^{ab'}$ ± 2.453	8.086^{a} ± 0.451
	Vit (E+C)	$9.102^{ac'}$ ± 1.023	0.993^{b} ± 0.114	$130.451^{ca'}$ ± 1.014	$6.991^{ca'}$ ± 0.401

Number of experimental rats is indicated in the parenthesis. a = $p<0.02$, b = $p<0.05$, c = $p<0.001$ as compared with controls, where as a' = $p<0.02$, b' = $p<0.05$ and c' = $p<0.001$ as compared to stressed rats.

Table-20. **Effect of treatment of Vitamin A, E and C individually and in combination (vitamin E + C) on Immobilization stress induced changes in Heart tissue levels of total and free SH groups.**

		GSH µmoles/g tissue	Free GSH µmoles/g tissue
Controls (10)		2.12 ± 0.01	0.091 ± 0.01
Stressed (10)		0.13^{c} ± 0.05	0.012^{b} ± 0.02
Pre-vitamin stressed treated (10) each	Vit A	$0.45^{bb'}$ ± 0.06	$0.02^{ba'}$ ± 0.012
	Vit E	$0.94^{bc'}$ ± 0.054	0.055^{b} ± 0.031
	Vit C	$0.72^{cc'}$ ± 0.121	$0.041^{ba'}$ ± 0.062
	Vit (E+C)	$1.13^{ba'}$ ± 0.432	0.064^{b} ± 0.012
Post-vitamin stressed treated (10) each	Vit A	0.83^{c} ± 0.241	$0.041^{ba'}$ ± 0.014
	Vit E	$1.96^{ab'}$ ± 0.632	$0.071^{ac'}$ ± 0.065
	Vit C	1.01^{b} ± 0.351	0.062^{a} ± 0.031
	Vit (E+C)	$2.01^{cc'}$ ± 0.214	$0.081^{ac'}$ ± 0.012

Number of experimental rats is indicated in the parenthesis. a = $p<0.02$, b = $p<0.05$, c = $p<0.001$ as compared with controls, where as a' = $p<0.02$, b' = $p<0.05$ and c' = $p<0.001$ as compared to stressed rats.

Table-21. **Effect of treatment of Vitamin A, E and C individually and in combination (vitamin E + C) on Immobilization stress induced changes in Heart tissue levels of ALP, GOT and GPT.**

		ALP nmol/min/mg of protein	GOT Units	GPT Units
Controls (10)		0.745 ± 0.012	55.161 ± 1.210	42.012 ± 0.912
Stressed (10)		2.456^{c} ± 0.013	81.426^{c} ± 1.012	69.152^{b} ± 0.123
Pre-vitamin stressed treated (10) each	Vit A	2.104^{b} ± 0.032	$57.069^{bc'}$ ± 0.195	$65.015^{ba'}$ ± 1.015
	Vit E	1.103^{b} ± 0.031	44.486^{a} ± 1.045	$52.019^{ab'}$ ± 0.214
	Vit C	$1.452^{bc'}$ ± 0.085	$49.148^{bc'}$ ± 0.145	56.047^{a} ± 2.015
	Vit (E+C)	$1.024^{ba'}$ ± 0.065	39.134^{a} ± 1.014	$45.197^{bc'}$ ± 0.459
Post-vitamin stressed treated (10) each	Vit A	$1.632^{bc'}$ ± 0.047	$66.158^{ca'}$ ± 1.452	$76.166^{bc'}$ ± 1.555
	Vit E	0.932^{c} ± 0.042	58.145^{c} ± 0.151	61.132^{b} ± 2.019
	Vit C	$1.232^{bb'}$ ± 0.054	$61.426^{ca'}$ ± 1.012	$75.136^{aa'}$ ± 1.046
	Vit (E+C)	$0.824^{ca'}$ ± 0.031	48.161^{c} ± 1.210	$59.012^{ca'}$ ± 2.012

Number of experimental rats is indicated in the parenthesis. a = $p<0.02$, b = $p<0.05$, c = $p<0.001$ as compared with controls, where as a' = $p<0.02$, b' = $p<0.05$ and c' = $p<0.001$ as compared to stressed rats.

Table-22. **Effect of treatment of Vitamin A, E and C individually and in combination (vitamin E + C) on Immobilization stress induced changes in Spleen tissue levels of SOD, Catalase, GST and MDA.**

		SOD Unit/mg protein	Catalase Unit/mg protein	GST nmoles/mg protein	MDA nmoles/mg of protein
Controls (10)		6.031 ± 0.932	2.965 ± 0.021	89.328 ± 3.021	1.322^{c} ± 0.645
Stressed rats (10)		1.965^{c} ± 0.011	0.865^{c} ± 0.165	35.255^{c} ± 4.011	4.324 ± 0.356
Pre-vitamin stressed treated (10) each	Vit A	$2.541^{ba'}$ ± 0.621	1.123^{b} ± 0.321	42.328^{a} ± 1.232	$1.933^{ca'}$ ± 0.652
	Vit E	$4.013^{bb'}$ ± 0.213	$2.018^{ac'}$ ± 0.621	66.521^{b} ± 1.329	3.155^{a} ± 0.321
	Vit C	3.121^{c} ± 0.921	1.658^{b} ± 0.641	59.692^{b} ± 5.123	$2.611^{ba'}$ ± 0.621
	Vit (E+C)	$4.910^{ba'}$ ± 0.301	$2.119^{ac'}$ ± 0.21	63.218^{b} ± 1.641	$3.219^{ac'}$ ± 0.352
Post-vitamin stressed treated (10) each	Vit A	3.560^{c} ± 0.321	1.629^{c} ± 0.412	49.651^{b} ± 3.621	$2.538^{ba'}$ ± 0.631
	Vit E	$5.311^{ba'}$ ± 0.321	$2.564^{aa'}$ ± 0.430	78.011^{a} ± 0.932	$3.976^{aa'}$ ± 0.329
	Vit C	$4.124^{cb'}$ ± 0.354	2.014^{a} ± 0.532	62.346^{b} ± 1.065	3.017^{b} ± 1.01
	Vit (E+C)	$5.913^{ac'}$ ± 0.821	$2.818^{ca'}$ ± 0.352	82.340^{a} ± 0.365	$4.106^{ac'}$ ± 0.901

Number of experimental rats is indicated in the parenthesis. a = $p<0.02$, b = $p<0.05$, c = $p<0.001$ as compared with controls, where as a' = $p<0.02$, b' = $p<0.05$ and c' = $p<0.001$ as compared to stressed rats.

Table-23. **Effect of treatment of Vitamin A, E and C individually and in combination (vitamin E + C) on Immobilization stress induced changes in Spleen tissue levels of total GSH, Free GSH and ALP.**

		GSH µmoles/g tissue	Free GSH µmoles/g tissue	ALP nmol/min/ mg of protein
Controls (10)		6.141 ± 0.99	4.522 ± 0.121	0.532 ± 0.001
Stressed (10)		1.013^{c} ± 0.68	1.215^{b} ± 0.921	2.442^{c} ± 0.043
Pre-Vitamin stressed treated (10) each	Vit A	$2.061^{bb'}$ ± 0.50	$1.518^{aa'}$ ±1.021	$2.130^{ab'}$ ± 0.093
	Vit E	$4.113^{ba'}$ ± 0.67	$2.686^{ba'}$ ± 0.941	$1.625^{ba'}$ ± 0.054
	Vit C	3.633^{a} ± 1.09	$2.214^{bc'}$ ± 1.11	$1.954^{ba'}$ ± 0.062
	Vit (E+C)	$4.512^{ba'}$ ± 0.87	3.818^{a} ± 0.624	$1.465^{bb'}$ ± 0.054
Post-vitamin stressed treated (10) each	Vit A	$2.796^{ca'}$ ± 1.04	$1.926^{ca'}$ ± 0.601	1.123^{c} ± 0.321
	Vit E	$5.917^{bc'}$ ± 1.35	3.946^{a} ± 0.634	$0.832^{cb'}$ ± 0.251
	Vit C	5.123^{b} ± 0.49	$3.018^{ab'}$ ± 0.621	$0.758^{ca'}$ ± 0.032
	Vit (E+C)	$6.422^{ab'}$ ± 1.23	$4.321^{ab'}$ ± 1.032	$0.632^{aa'}$ ± 0.065

Number of experimental rats is indicated in the parenthesis. a = $p<0.02$, b = $p<0.05$, c = $p<0.001$ as compared with controls, where as a' = $p<0.02$, b' = $p<0.05$ and c' = $p<0.001$ as compared to stressed rats.

Table-24. **Effect of treatment of Vitamin A, E and C individually and in combination (vitamin E + C) on Immobilization stress induced changes in Kidney tissue levels of SOD, Catalase, GST and MDA.**

		SOD Unit/mg protein	Catalase Unit/mg protein	GST nmoles/mg protein	MDA nmoles/mg of protein
Controls (10)		12.011 ± 0.023	8.911 ± 1.023	164.321 ± 3.021	5.320 ± 0.421
Stressed rats (10)		3.115^{c} ± 0.164	2.916^{c} ± 2.012	99.326^{c} ± 3.511	14.326^{c} ± 1.021
Pre-vitamin stress treated (10) each	Vit A	$4.313^{ab'}$ ± 0.921	$3.564^{cc'}$ ± 0.215	$110.326^{ba'}$ ± 4.321	$12.394^{ab'}$ ± 0.942
	Vit E	$7.134^{ba'}$ ± 0.301	$6.232^{aa'}$ ± 0.935	$135.621^{cb'}$ ± 2.013	9.014^{b} ± 1.012
	Vit C	$5.315^{cb'}$ ± 0.932	4.024^{b} ± 1.321	120.316^{c} ± 1.021	$11.023^{ca'}$ ± 0.924
	Vit (E+C)	8.314^{b} ± 0.612	$7.136^{ac'}$ ± 0.352	$140.325^{ba'}$ ± 1.952	$8.125^{ba'}$ ± 0.621
Post-vitamin stress treated (10) each	Vit A	$6.616^{ca'}$ ± 1.031	$4.964^{cc'}$ ± 1.210	$128.659^{ca'}$ ± 0.921	$10.621^{ba'}$ ± 0.673
	Vit E	10.610^{b} ± 0.621	7.625^{a} ± 1.023	152.620^{a} ± 4.023	6.371^{a} ± 1.920
	Vit C	$8.215^{bc'}$ ± 1.321	$6.349^{ab'}$ ± 0.932	$132.841^{cb'}$ ± 2.39	$8.093^{ba'}$ ± 0.213
	Vit (E+C)	$11.323^{ac'}$ ± 0.432	$8.364^{ac'}$ ± 0.432	$155.654^{ab'}$ ± 0.321	6.024^{a} ± 2.011

Number of experimental rats is indicated in the parenthesis. a = $p<0.02$, b = $p<0.05$, c = $p<0.001$ as compared with controls, where as a' = $p<0.02$, b' = $p<0.05$ and c' = $p<0.001$ as compared to stressed rats.

Table-25. **Effect of treatment of Vitamin A, E and C individually and in combination (vitamin E + C) on Immobilization stress induced changes in Kidney tissue levels of total GSH, Free GSH and ALP.**

		GSH µmoles/g tissue	Free GSH µmoles/g tissue	ALP nmol/min/mg of protein
Controls (10)		5.322 ± 0.932	1.351 ± 0.032	1.191 ± 0.012
Stressed (10)		1.234^{c} ± 1.012	0.385^{c} ± 0.086	3.012^{b} ± 0.032
Pre-vitamin stressed treated (10) each	Vit A	$2.124^{bb'}$ ± 0.324	0.596^{c} ± 0.064	$2.661^{ac'}$ ± 0.054
	Vit E	3.244^{b} ± 1.231	$0.934^{bb'}$ ± 0.091	1.932^{a} ± 0.014
	Vit C	$3.016^{bc'}$ ± 0.954	0.726^{b} ± 0.011	2.032^{a} ± 0.062
	Vit (E+C)	4.124^{a} ± 0.532	$1.014^{ba'}$ ± 0.063	$1.865^{ab'}$ ± 0.021
Post-vitamin stressed treated (10) each	Vit A	3.358^{b} ± 0.365	0.834^{c} ± 0.05	$2.621^{ab'}$ ± 0.075
	Vit E	4.627^{a} ± 0.354	$1.236^{ab'}$ ± 0.214	1.532^{b} ± 0.006
	Vit C	$3.523^{ba'}$ ± 1.321	1.114^{a} ± 0.120	$1.562^{ba'}$ ± 0.214
	Vit (E+C)	5.218^{a} ± 0.624	$1.316^{aa'}$ ± 0.214	1.204^{c} ± 0.214

Number of experimental rats is indicated in the parenthesis. a = $p<0.02$, b = $p<0.05$, c = $p<0.001$ as compared with controls, where as a' = $p<0.02$, b' = $p<0.05$ and c' = $p<0.001$ as compared to stressed rats.

Table-26. **Effect of high dose of vitamin A and E on Immobilization stress induced changes in Liver tissue levels of SOD, catalase, GST and MDA.**

	SOD Unit/mg protein	Catalase Unit/mg protein	GST nmoles/mg protein	MDA nmoles/mg of protein
Control (10)	15.322 ± 0.214	10.365 ± 0.365	162.032 ± 6.021	8.023 ± 0.365
Stressed (10)	9.036^{c} ± 0.351	6.325^{c} ± 0.215	123.024^{c} ± 3.025	15.023^{c} ± 0.325
Vitamin A (10)	16.025 ± 0.532	9.025 ± 0.251	161.265 ± 5.026	7.265 ± 0.254
Vitamin E (10)	17.021 ± 0.652	10.532 ± 0.568	170.263 ± 3.021	7.010 ± 0.154
Post-vitamin A stressed treated (10)	$11.265^{bc'}$ ± 0.362	$8.025^{bc'}$ ± 1.025	$135.021^{ca'}$ ± 5.021	$12.325^{bc'}$ ± 0.215
Post-vitamin E stressed treated (10)	$13.563^{cb'}$ ± 0.563	$9.265^{ab'}$ ± 0.251	$153.021^{bc'}$ ± 4.021	$9.021^{ba'}$ ± 0.251

Number of experimental rats is indicated in the parenthesis. a = $p<0.02$, b = $p<0.05$, c = $p<0.001$ as compared with controls, where as a' = $p<0.02$, b' = $p<0.05$ and c' = $p<0.001$ as compared to stressed rats.

Table-27. **Effect of high dose of vitamin A and E on Immobilization stress induced changes in Liver tissue levels of total and free •SH groups.**

	GSH µmoles/g tissue	Free GSH µmoles/g tissue
Control (10)	15.023 ± 1.012	1.354 ± 0.012
Stressed (10)	9.110^{c} ± 0.925	0.521^{c} ± 0.012
Vitamin A (10)	14.365 ± 0.521	1.202 ± 0.05
Vitamin E (10)	16.025 ± 0.325	1.456 ± 0.032
Post-vitamin A stressed treated (10)	$11.325^{bc'}$ ± 0.531	$0.732^{ca'}$ ± 0.04
Post-vitamin E stressed treated (10)	$13.026^{ba'}$ ± 0.632	$1.215^{ca'}$ ± 0.01

Number of experimental rats is indicated in the parenthesis. a = $p<0.02$, b = $p<0.05$, c = $p<0.001$ as compared with controls, where as a' = $p<0.02$, b' = $p<0.05$ and c' = $p<0.001$ as compared to stressed rats.

Table-28. **Effect of high dose of vitamin A and E on Immobilization stress induced changes in Liver tissue levels of GOT GPT and ALP.**

	GOT Units	GPT Units	ALP nmol/min/mg of protein
Control (10)	29.321 ± 3.254	19.214 ± 2.314	0.665 ± 0.01
Stressed (10)	42.356^{c} ± 5.698	27.645^{b} ± 5.021	1.025^{c} ± 0.021
Vitamin A (10)	28.314 ± 4.201	17.325 ± 3.541	$0.852^{'}$ ± 0.021
Vitamin E (10)	27.521 ± 5.021	16.548 ± 2.512	0.932 ± 0.015
Post-vitamin A stressed treated (10)	$36.021^{aa'}$ ± 3.622	$26.124^{ba'}$ ± 2.651	$0.832^{ca'}$ ± 0.014
Post-vitamin E stressed treated (10)	$32.865^{cb'}$ ± 4.215	$21.654^{ab'}$ ± 2.654	$0.763^{ba'}$ ± 0.063

Number of experimental rats is indicated in the parenthesis. a = $p<0.02$, b = $p<0.05$, c = $p<0.001$ as compared with controls, where as a' = $p<0.02$, b' = $p<0.05$ and c' = $p<0.001$ as compared to stressed rats.

Table-29. **Effect of high dose of vitamin A and E on Immobilization stress induced changes in Brain levels of SOD, catalase, GST and MDA.**

	SOD Unit/mg protein	Catalase Unit/mg protein	GST nmoles/mg protein	MDA nmoles/mg of protein
Control (10)	5.032 ± 0.035	6.816 ± 0.254	125.362 ± 3.021	6.032 ± 0.125
Stressed (10)	3.215^{c} ± 0.056	4.125^{b} ± 0.235	98.035^{c} ± 4.032	10.254^{c} ± 0.632
Vitamin A (10)	6.325^{a} ± 0.075	7.021 ± 0.025	130.152^{a} ± 3.026	5.321+ ± 0.352
Vitamin E (10)	5.251 ± 0.431	7.235^{a} ± 0.054	136.652^{a} ± 3.026	5.065^{a} ± 0.254
Post-vitamin A stressed treated (10)	$4.031^{ac'}$ ± 0.315	$4.932^{bc'}$ ± 0.052	$113.523^{ba'}$ ± 3.025	$7.023^{bc'}$ ± 0.225
Post-vitamin E stressed treated (10)	$6.021^{ca'}$ ± 0.532	$6.302^{ac'}$ ± 0.521	$121.265^{aa'}$ ± 3.562	$8.635^{bc'}$ ± 0.521

Number of experimental rats is indicated in the parenthesis. a = $p<0.02$, b = $p<0.05$, c = $p<0.001$ as compared with controls, where as a' = $p<0.02$, b' = $p<0.05$ and c' = $p<0.001$ as compared to stressed rats.

Table-30. **Effect of high dose of vitamin A and E on Immobilization stress induced changes in Brain levels of total, free SH groups and ALP.**

	GSH µmoles/g tissue	Free GSH µmoles/g tissue	ALP nmol/min/mg of protein
Control (10)	13.254 ±1.032	2.365 ± 0.012	0.215 ± 0.001
Stressed (10)	7.365^{c} ± 0.932	0.863^{c} ± 0.001	0.532^{c} ± 0.052
Vitamin A (10)	14.265 ± 1.201	2.563 ± 0.036	0.251 ± 0.05
Vitamin E (10)	13.265 ± 0.235	2.865 ± 0.053	0.196 ± 0.061
Post-vitamin A stressed treated (10)	$9.025^{ba'}$ ± 0.458	$1.126^{ac'}$ ± 0.024	$0.963^{ba'}$ ± 0.055
Post-vitamin E stressed treated (10)	$11.023^{ca'}$ ± 0.563	$2.036^{bc'}$ ± 0.063	$0.432^{ba'}$ ± 0.01

Number of experimental rats is indicated in the parenthesis. a = $p<0.02$, b = $p<0.05$, c = $p<0.001$ as compared with controls, where as a' = $p<0.02$, b' = $p<0.05$ and c' = $p<0.001$ as compared to stressed rats.

Table-31. **Effect of high dose of vitamin A and E on Immobilization stress induced changes in Heart tissue levels of SOD, catalase, GST and MDA.**

	SOD Unit/mg protein	Catalase Unit/mg protein	GST nmoles/mg protein	MDA nmoles/mg of protein
Control (10)	11.632 ± 0.123	2.365 ± 0.012	127.325 ± 3.021	4.032 ± 0.214
Stressed (10)	7.325^{c} ± 0.321	1.152^{b} ± 0.215	102.321^{c} ± 4.023	7.625^{b} ± 0.015
Vitamin A (10)	13.214 ± 0.215	2.523 ± 0.254	129.021 ± 4.021	4.123 ± 0.021
Vitamin E (10)	14.025 ± 0.215	3.102 ± 0.125	135.021 ± 3.021	3.532 ± 0.025
Post vitamin A stressed treated (10)	8.963^{b} ± 1.02	$1.562^{ba'}$ ± 0.010	$115.021^{aa'}$ ± 3.219	6.321^{a} ± 0.032
Post vitamin E stressed treated (10)	$11.021^{cc'}$ ± 0.221	$2.521^{ba'}$ ± 0.065	$119.302^{ca'}$ ± 4.032	$4.625^{ca'}$ ± 0.346

Number of experimental rats is indicated in the parenthesis. a = $p<0.02$, b = $p<0.05$, c = $p<0.001$ as compared with controls, where as a' = $p<0.02$, b' = $p<0.05$ and c' = $p<0.001$ as compared to stressed rats

Table-32. **Effect of high dose of vitamin A and E on Immobilization stress induced changes in Heart tissue levels of total and free SH groups.**

	GSH µmoles/g tissue	Free GSH µmoles/g tissue
Control (10)	5.362 ± 0.152	1.021 ± 0.05
Stressed (10)	3.120^{b} ± 0.012	0.352^{b} ± 0.04
Vitamin A (10)	5.496 ± 0.325	1.123^{-} ± 0.031
Vitamin E (10)	6.563 ± 0.254	1.302 ± 0.065
Post-vitamin A stressed treated (10)	$4.632^{aa'}$ ± 0.021	$0.532^{bc'}$ ± 0.063
Post-vitamin E stressed treated (10)	$6.021^{ca'}$ ± 0.321	$0.865^{bb'}$ ± 0.045

Number of experimental rats is indicated in the parenthesis. a = $p<0.02$, b = $p<0.05$, c = $p<0.001$ as compared with controls, where as a' = $p<0.02$, b' = $p<0.05$ and c' = $p<0.001$ as compared to stressed rats.

Table-33. **Effect of high dose of vitamin A and E on Immobilization stress induced changes in Heart tissue levels of GOT, GPT and ALP.**

	GOT Units	GPT Units	ALP nmol/min/mg of protein
Control (10)	35.125 ± 3.215	24.325 ± 3.214	0.446 ± .012
Stressed (10)	44.251^{c} ± 5.321	36.215^{b} ± 2.144	1.235^{c} ± 0.025
Vitamin A (10)	33.215 ± 6.021	23.251 ± 3.156	0.392 ± 0.053
Vitamin E (10)	34.251 ± 2.015	22.631 ± 2.154	0.352 ± 0.034
Post-vitamin A stressed treated (10)	$41.521^{aa'}$ ± 4.521	$31.665^{ba'}$ ± 3.568	$0.932^{bc'}$ ± 0.016
Post-vitamin E stressed treated (10)	$37.021^{cc'}$ ± 4.214	$26.562^{ca'}$ ± 4.512	$0.621^{ba'}$ ± 0.021

Number of experimental rats is indicated in the parenthesis. a = $p<0.02$, b = $p<0.05$, c = $p<0.001$ as compared with controls, where as a' = $p<0.02$, b' = $p<0.05$ and c' = $p<0.001$ as compared to stressed rats.

Table-34. **Effect of high dose of vitamin A and E on Immobilization stress induced changes in Spleen tissue levels of SOD, catalase, GST and MDA.**

	SOD Unit/mg protein	Catalase Unit/mg protein	GST nmoles/mg protein	MDA nmoles/mg of protein
Control (10)	8.265 ± 0.052	4.362 ± 0.032	90.625 ± 2.325	1.123^{a} ± 0.053
Stressed (10)	4.215^{c} ± 0.032	2.136^{b} ± 0.042	68.021^{c} ± 3.265	1.954 ± 0.036
Vitamin A (10)	8.312 ± 1.021	4.632 ± 1.023	95.325 ± 4.023	2.062 ± 0.063
Vitamin E (10)	9.432 ± 0.521	5.326 ± 0.021	101.326 ± 5.021	2.632 ± 0.251
Post-vitamin A stressed treated (10)	$5.321^{bc'}$ ± 0.125	3.256^{b} ± 0.053	$73.265^{ca'}$ ± 2.023	$2.136^{bc'}$ ± 0.021
Post-vitamin E stressed treated (10)	$8.325^{ca'}$ ± 0.210	$4.965^{ba'}$ ± 0.095	$84.021^{bc'}$ ± 3.026	1.532^{a} ± 0.036

Number of experimental rats is indicated in the parenthesis. a = $p<0.02$, b = $p<0.05$, c = $p<0.001$ as compared with controls, where as a' = $p<0.02$, b' = $p<0.05$ and c' = $p<0.001$ as compared to stressed rats.

Table-35. **Effect of high dose of vitamin A and E on Immobilization stress induced changes Spleen tissue levels of total, free SH groups and ALP.**

	GSH µmoles/g tissue	Free GSH µmoles/g tissue	ALP nmol/min/mg of protein
Control (10)	11.952 ± 1.036	2.689 ± 0.032	0.125 ± 0.031
Stressed (10)	7.562^{c} ± 0.042	1.232^{b} ± 0.032	0.0721^{c} ± 0.001
Vitamin A (10)	11.025 ± 0.852	2.798` ± 0.063	0.135 ± 0.015
Vitamin E (10)	12.035 ± 0.452	3.105 ± 0.022	0.210 ± 0.002
Post-vitamin A stressed treated (10)	$8.326^{ba'}$ ± 0.532	1.765^{b} ± 0.152	$0.149^{cb'}$ ± 0.01
Post-vitamin E stressed treated (10)	$12.325^{aa'}$ ± 0.965	$2.536^{ba'}$ ± 0.215	$0.093^{ac'}$ ± 0.012

Number of experimental rats is indicated in the parenthesis. a = $p<0.02$, b = $p<0.05$, c = $p<0.001$ as compared with controls, where as a' = $p<0.02$, b' = $p<0.05$ and c' = $p<0.001$ as compared to stressed rats.

Table-36. **Effect of high dose of vitamin A and E on Immobilization stress induced changes in Kidney tissue levels of SOD, catalase, GST and MDA.**

	SOD Unit/mg protein	Catalase Unit/mg protein	GST nmoles/mg protein	MDA nmoles/mg of protein
Control (10)	13.265 ± 1.032	7.965 ± 0.112	149.025 ± 6.032	3.125 ± 0.053
Stressed (10)	8.325^{c} ± 0.421	3.452^{c} ± 0.021	118.325^{c} ± 4.325	6.421^{b} ± 0.324
Vitamin A (10)	13.921 ± 0.321	8.062 ± 0.214	144.632 ± 3.965	3.048 ± 0.214
Vitamin E (10)	14.532 ± 1.032	8.732 ± 0.251	151.362 ± 4.021	2.951 ± 0.032
Post-vitamin A stressed treated (10)	10.214^{b} ± 0.214	4.658^{c} ± 0.245	129.366^{b} ± 5.032	5.124^{c} ± 0.321
Post-vitamin E stressed treated (10)	$12.365^{aa'}$ ± 0.932	$6.935^{bc'}$ ± 0.521	$138.651^{bb'}$ ± 5.125	$2.935^{ac'}$ ± 0.013

Number of experimental rats is indicated in the parenthesis. a = $p<0.02$, b = $p<0.05$, c = $p<0.001$ as compared with controls, where as a' = $p<0.02$, b' = $p<0.05$ and c' = $p<0.001$ as compared to stressed rats.

Table-37. **Effect of high dose of vitamin A and E on Immobilization stress induced changes in Kidney tissue levels of total, free SH groups and ALP.**

	GSH µmoles/g tissue	Free GSH µmoles/g tissue	ALP nmol/min/mg of protein
Control (10)	9.021 ± 0.219	2.325 ± 0.021	0.832 ± 0.052
Stressed (10)	5.321^{c} ± 0.125	1.124^{b} ± 0.036	1.421^{b} ± 0.072
Vitamin A (10)	10.214 ± 0.351	2.391 ± 0.215	0.812 ± 0.038
Vitamin E (10)	12.321 ± 0.521	3.124 ± 0.038	0.632 ± 0.112
Post-vitamin A stressed treated (10)	$6.521^{aa'}$ ± 0.321	1.532^{ba} ± 0.057	$1.021^{ac'}$ ± 0.026
Post-vitamin E stressed treated (10)	$8.932^{bc'}$ ± 0.251	$2.521^{ca'}$ ± 0.021	$0.721^{ca'}$ ± 0.061

Number of experimental rats is indicated in the parenthesis. a = $p<0.02$, b = $p<0.05$, c = $p<0.001$ as compared with controls, where as a' = $p<0.02$, b' = $p<0.05$ and c' = $p<0.001$ as compared to stressed rats.

c). The restraint stress induced decrease in the levels of total SH groups and glucose were found significantly enhanced ($p<0.05$, $p<0.05$ respectively) by the extract treatment as compared to stressed rats (Fig-2c, 3b).

The levels of MDA ($p<0.05$) and uric acid ($p<0.05$) were observed significantly decreased by the pre-extract stress treatment of *S. nigrum* as compared to stress alone treatments (Fig-1d, 2d).

The levels of marker enzymes like SALP ($p<0.02$), SGOT ($p<0.05$) and SGPT ($p<0.01$) were found significantly decreased in the pre-extract stressed groups when a comparison was made with stress treated rats (Fig-2a, b, 3a).

In the post-extract stress treatment, the circulating activities of free radical scavenging enzymes like SOD ($p<0.001$), GST ($p<0.02$) and catalase ($p<0.001$) were found significantly increased as compared to stress treated rats, but still remained significantly lower than the control values (Fig-1 a,b,c).

The treatment of aqueous extract of *S. nigrum* leaves after the stress exposure (post-extract stress treatment) resulted in a significant decrease in the levels of MDA ($p<0.001$) and uric acid ($p<0.001$) (Fig-1d, 2d) as compared to stress alone treated rats, whereas the levels of total SH groups ($p<0.001$) and glucose ($p<0.02$) were observed significantly increased in the post-extract stress treatment when a comparison was made with their respective control (Fig-2c, 3b).

The restraint stress induced increased levels of SALP, SGOT and SGPT were found significantly decreased ($p<0.001$, $p<0.001$, $p<0.02$ respectively) by the extract treatment after the stress exposure as compared to their respective control values (Fig-2a, b, 3a).

Though, the pattern of change in these parameters was found same in all the tissues like liver, brain, heart, spleen and kidney; but the maximum antioxidant effect of the extract was observed in the liver while minimum in the heart tissues.

In the liver tissues, the post-extract stress treatment resulted in a significant increase in the circulating activities of SOD ($p<0.02$), GST ($p<0.05$) and catalase ($p<0.02$) as compared to stress alone treatment (Fig-4a,b,c). The restraint stress induced decreased levels of total SH groups and glucose were found significantly enhanced ($p<0.001$, $p<0.05$) by the post-extract stress treatment (Fig-5a,d).

The liver levels of MDA ($p<0.001$) and uric acid ($p<0.02$) were found significantly decreased by the post-extract stress treatment as compared to stress treated rats (Fig-4d, 5b).

Extract treatments either prior to or after stress resulted in a significant decrease in the liver marker enzymes like ALP ($p<0.001$), GOT ($p<0.05$) and GPT ($p<0.001$) as compared to stress alone treated rats and a reversion towards control values was observed (Fig-5c, 6a, b).

In the heart tissues, the restraint stress induced decreased activities of SOD, GST and catalase were found significantly increased ($p<0.01$, $p<0.02$, $p<0.01$ respectively) by the post-extract stress treatment as compared to stressed rats (Fig-7a,b,c).The enhanced levels of uric acid and MDA in the heart tissues by restraint stress were significantly ($p<0.05$, $p<0.001$) decreased by the treatment of *S. nigrum* leaves extract, whereas the levels of total SH groups ($p<0.001$) and glucose ($p<0.001$) were found significantly increased when a comparison was made with stress alone treated rats (Fig-7d, 8a,b,d).

The heart levels of marker enzymes like ALP, GOT and GPT were found significantly decreased ($p<0.05$. $p<0.001$, $p<0.05$ respectively) by the post extract stress treatment (Fig 8c, 9a,b).

The pattern of change in these parameters by extract treatments was observed nearly the same in other tissues like liver, brain, heart, spleen and kidney, though the extent of variation was different which can be summarized as follows

Liver > Kidney > Brain > Spleen > Heart

i.e. maximum variations from controls / stress were observed in liver tissues while minimum in heart tissues; though the changes observed were significant.

The treatment of extract either prior to or after the restraint stress exposure resulted in significant reversion of free radical scavenging enzyme activities and the levels of other biochemical parameters towards their respective control values. However, the post-extract treatment showed a significant reversion of the stress altered biochemical changes towards their respective normal control values than the pre-extract treatment.

Effect of aqueous extract of onion and garlic on restraint stress induced oxidative changes in rat plasma and tissues

A single oral dose of crude extracts of garlic and onion (100 mg/kg bw) was given to rats both before (pre-extract stress treatment) and after (post-extract stress treatment) 6 h of stress exposure as described in methods.

The treatment of onion / garlic extract prior to stress was found effective in resisting the restraint stress induced oxidative changes in rats. However, the pre-garlic extract treatment was found more effective than onion treatment in resisting the alteration of measured biochemical parameters due to stress. The pre-treatment of extracts caused a significant enhancement in the circulating activities of free radical scavenging enzymes i.e. SOD, GST and catalase as compared to stress alone treatments, ($p<0.05$, $p<0.05$, $p<0.02$ respectively for onion; and $p<0.05$, $p<0.02$, $p<0.02$ respectively for garlic) (Fig 16a,b,c).

The levels of glucose, total and free SH groups were found significantly ($p<0.02$) increased by the onion / garlic treatment prior to stress exposure, when a comparison was made with the stressed rats. (Fig 17c, 18b,c), whereas the levels of MDA and uric acid were observed significantly ($p<0.05$) decreased by these treatments as compared to stress alone treatment (Fig 16d, 17d).

As compared to stressed rats the levels of marker enzymes like SALP, SGOT and SGPT ($p<0.02$ for onion treatments) were found significantly decreased by the treatment of both onion and garlic extracts before the stress exposure ($p<0.05$, $p<0.02$, $p<0.02$ respectively for the garlic treatments) (Fig 17a,b, 18a).

Significant reversion in the oxidative stress parameters towards control values was observed by the post extract than pre-extract treatments.

In the post-onion / garlic extract treatments, the circulating activities of free radical scavenging enzymes like SOD ($p<0.001$), GST ($p<0.02$) and catalase ($p<0.05$) were found significantly increased as compared to either pre-extract stress treatments of both the extracts or stress alone treatment (Fig 16a,b,c).

The restraint stress induced increased levels of MDA and uric acid were found significantly decreased by the post-onion / garlic extract stress treatments as compared to stress alone treatment, the significance levels of both the treatments are $p<0.02$, $p<0.001$ respectively for onion and ($p<0.001$, $p<0.001$ respectively for garlic (Fig 16d, 17d), whereas the levels of total, free SH groups and glucose were observed significantly ($p<0.05$, $p<0.001$ respectively) increased by the post-onion / garlic extract stress treatments, when a comparison was made with stress alone treatment (Fig 17c, 18b,c).

The levels of marker enzymes i.e. SALP ($p<0.02$, $p<0.001$), SGOT ($p<0.02$, $p<0.001$) and SGPT ($p<0.05$, $p<0.05$) were found significantly decreased in the post-extract stress treatment of both the extracts (onion / garlic) as compared to stressed rats (Fig 17a,b, 18a).

The antioxidant effect of both the extracts was found maximum in liver while minimum in spleen tissues in terms of measured biochemical parameters.

In the liver tissues, the restraint stress induced decreased activities of free radical scavenging enzymes like SOD, GST and catalase were found significantly increased ($p<0.001$, $p<0.001$, $p<0.001$ respectively) by the

treatment of onion / garlic extracts after the stress exposure when a comparison was made with the stressed rats (Fig-19a,b,c).

Garlic extract treatment after the stress exposure caused a significant increase in the liver level of glucose ($p<0.05$), total and free SH groups ($p<0.05$) (Fig-20c, 21b,c), whereas the levels of MDA ($p<0.001$) and uric acid ($p<0.001$) were found significantly decreased as compared to stress alone treatment (Fig-19d, 20d).

The increased liver levels of marker enzymes like ALP, GOT and GPT due to restraint stress were found significantly decreased ($p<0.02$, $p<0.001$, $p<0.001$ respectively) by the post-garlic extract treatment as compared to stress alone treatment (Fig-20a,b, 21a).

In spleen the tissues activities of SOD ($p<0.05$), GST ($p<0.05$) and catalase ($p<0.001$) was found significantly increased by the post treatment of garlic extract as compared to stress treated rats (Fig-22a,b,c), but the changes were less marked than liver tissues.

The restraint stress induced increase in the spleen levels of MDA and uric acid were found significantly decreased ($p<0.001$, $p<0.05$ respectively) (Fig-22d, 23a) by the post treatment of garlic, whereas the levels of glucose, total and free SH ($p<0.05$) groups were significantly increased when a comparison was made with restraint stress treated rats (Fig-28b,d,e).

The spleen tissue level of marker enzyme ALP ($p<0.001$) was found significantly decreased by the post-garlic extract treatment as compared to stressed rats (Fig-28e).

The garlic and onion extract treatments both prior to and after restraint stress were found effective in restoring the stress induced alteration of the above mentioned biochemical parameters towards their respective control values. However, the post-garlic extract treatment was found most effective than pre-garlic pre / post onion extract treatments.

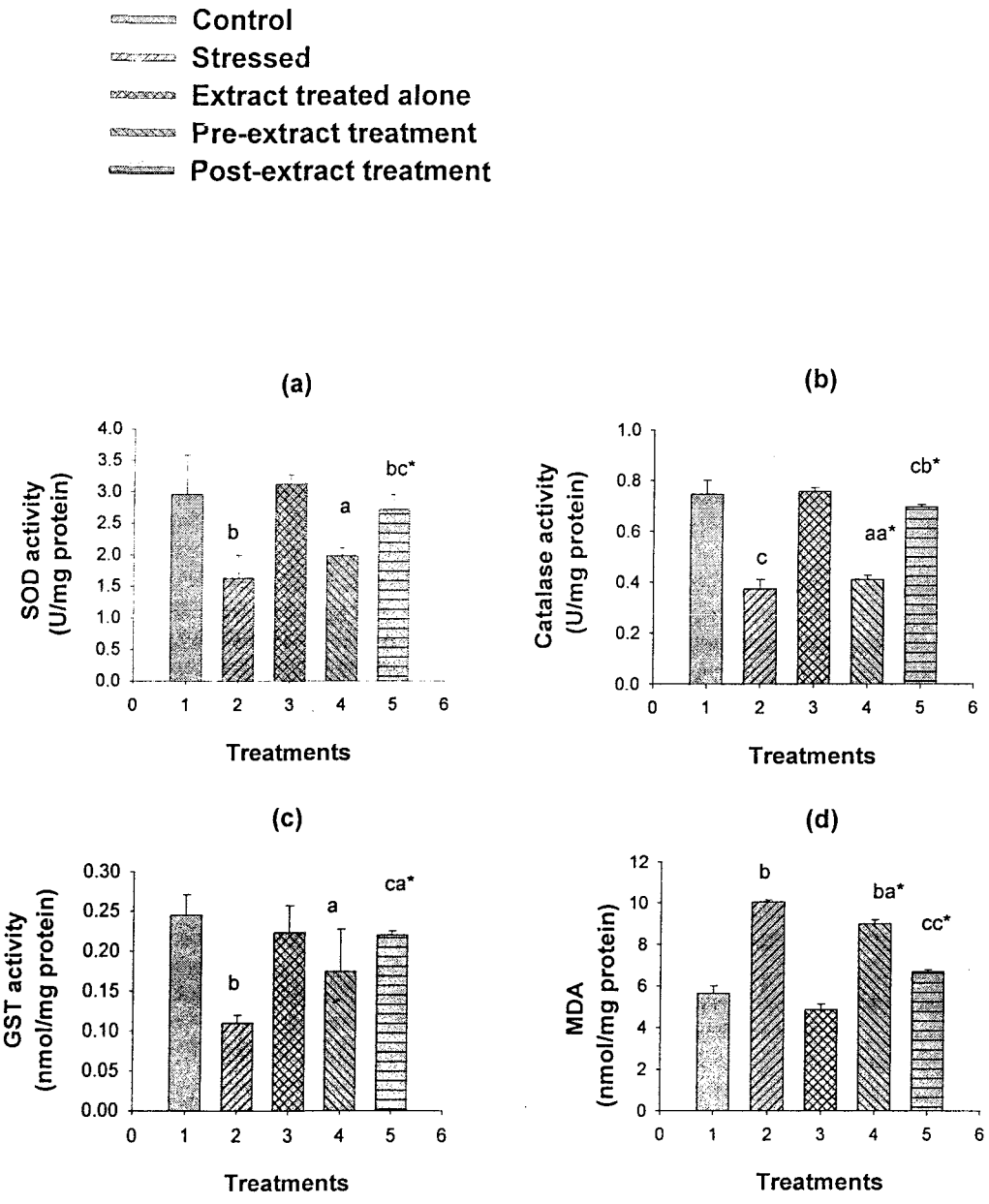
The pattern of change in these parameters by both the extract treatments was observed nearly the same in the other tissues like liver, heart,

brain, spleen and kidney, though the extent of variation was different which can be summarized as follows:

Liver > Kidney > Heart > Brain > Spleen

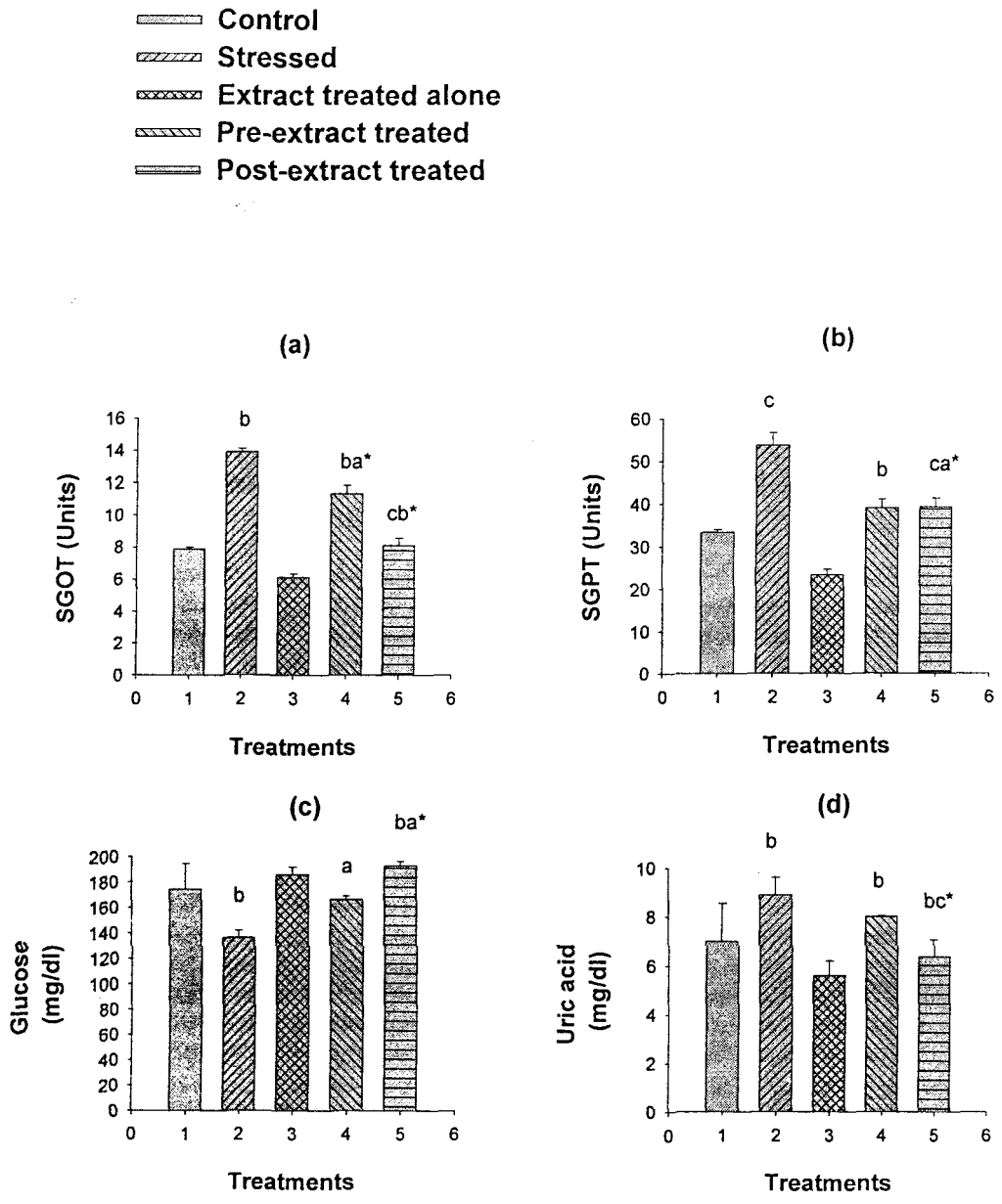
In all the tissues garlic extract was found more effective in restoring the altered parameters towards control values than onion extract.

Fig-1 Effect of crude extract of *S. nigrum* on immobilization stress induced changes in circulating levels of SOD, Catalase, GST and MDA



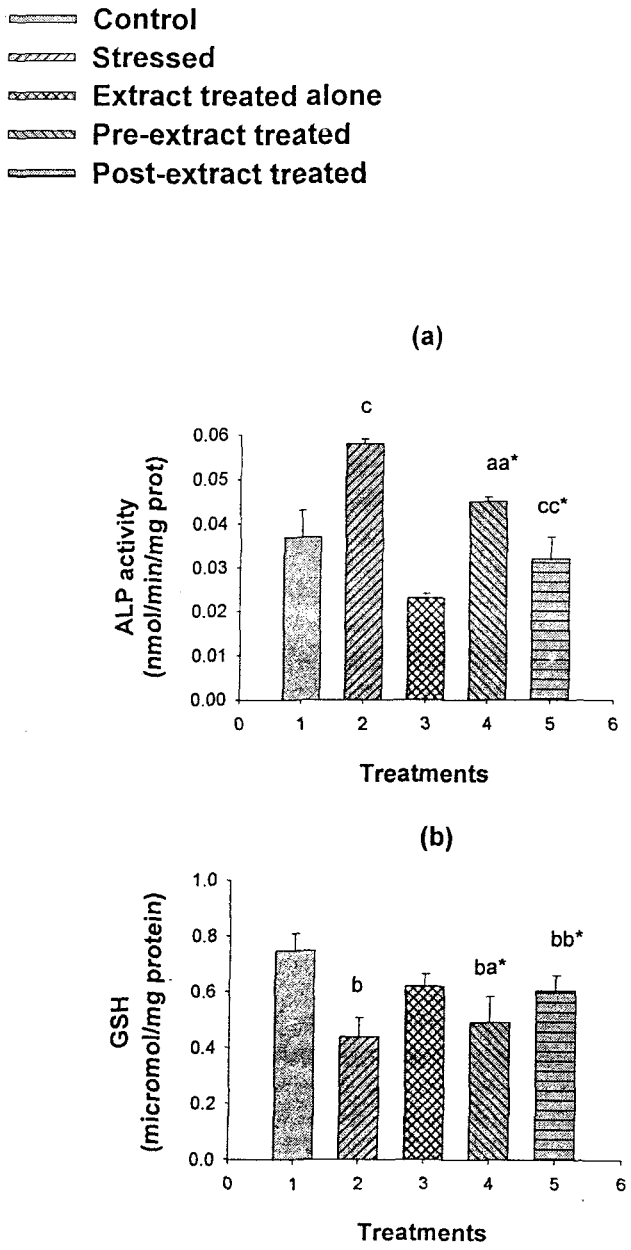
a = p<0.02, b = p<0.05 and c = p<0.001 shows p value as compared to controls where as a* = p<0.02, b* = p<0.05 and c* = p<0.001 shows p values as compared to stressed rats.

Fig- 2. Effect of crude extract of *S. nigrum* on immobilization stress Induced changes in circulating levels of SGOT, SGPT, Glucose and Uric acid



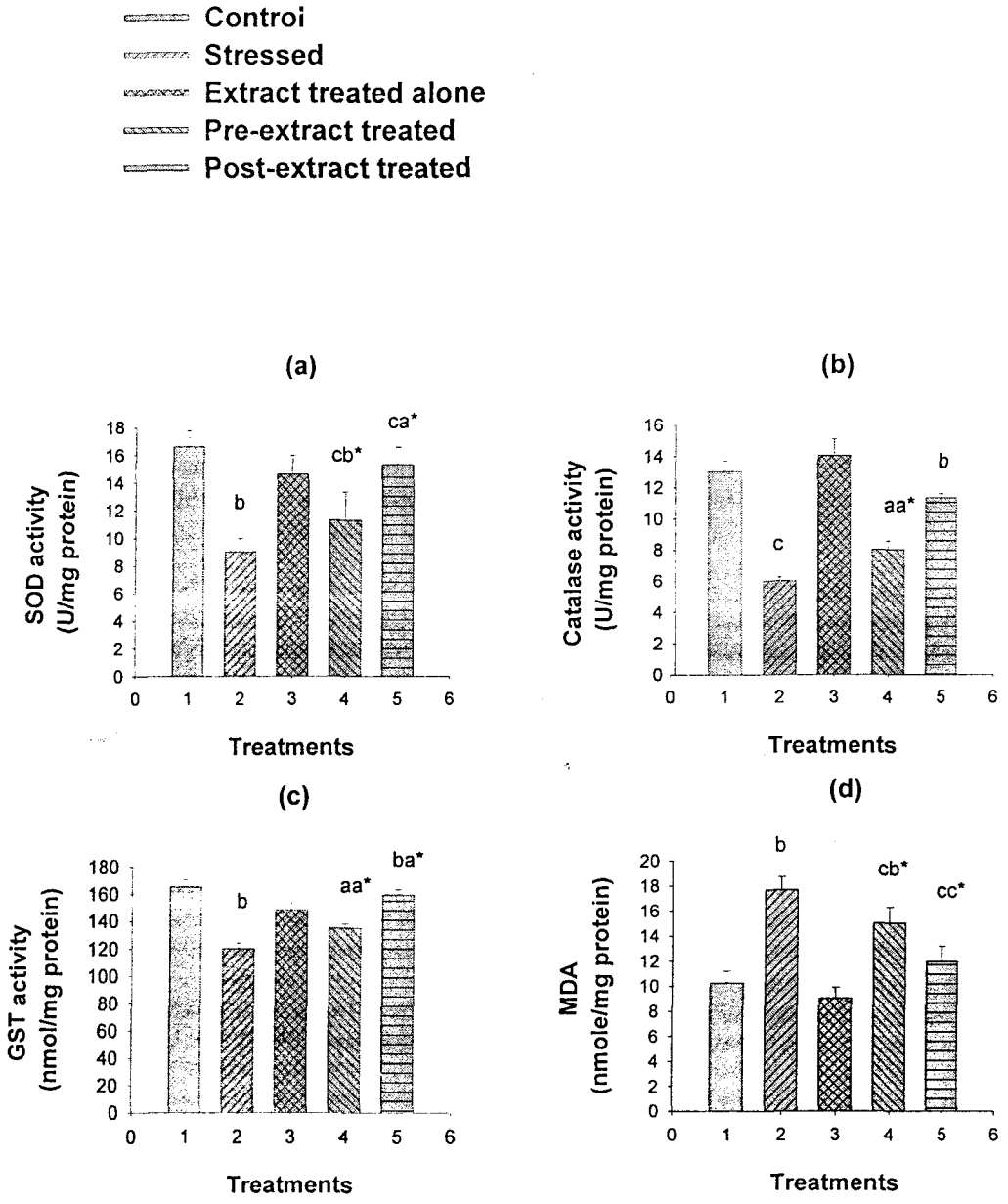
a = p<0.02, b = p<0.05 and c = p<0.001 shows p value as compared to controls where as a* = p<0.02, b* = p<0.05 and c* = p<0.001 shows p values as compared to stressed rats.

Fig- 3. Effect of crude extract of *S. nigrum* on immobilization stress induced changes in circulating levels of ALP and GSH



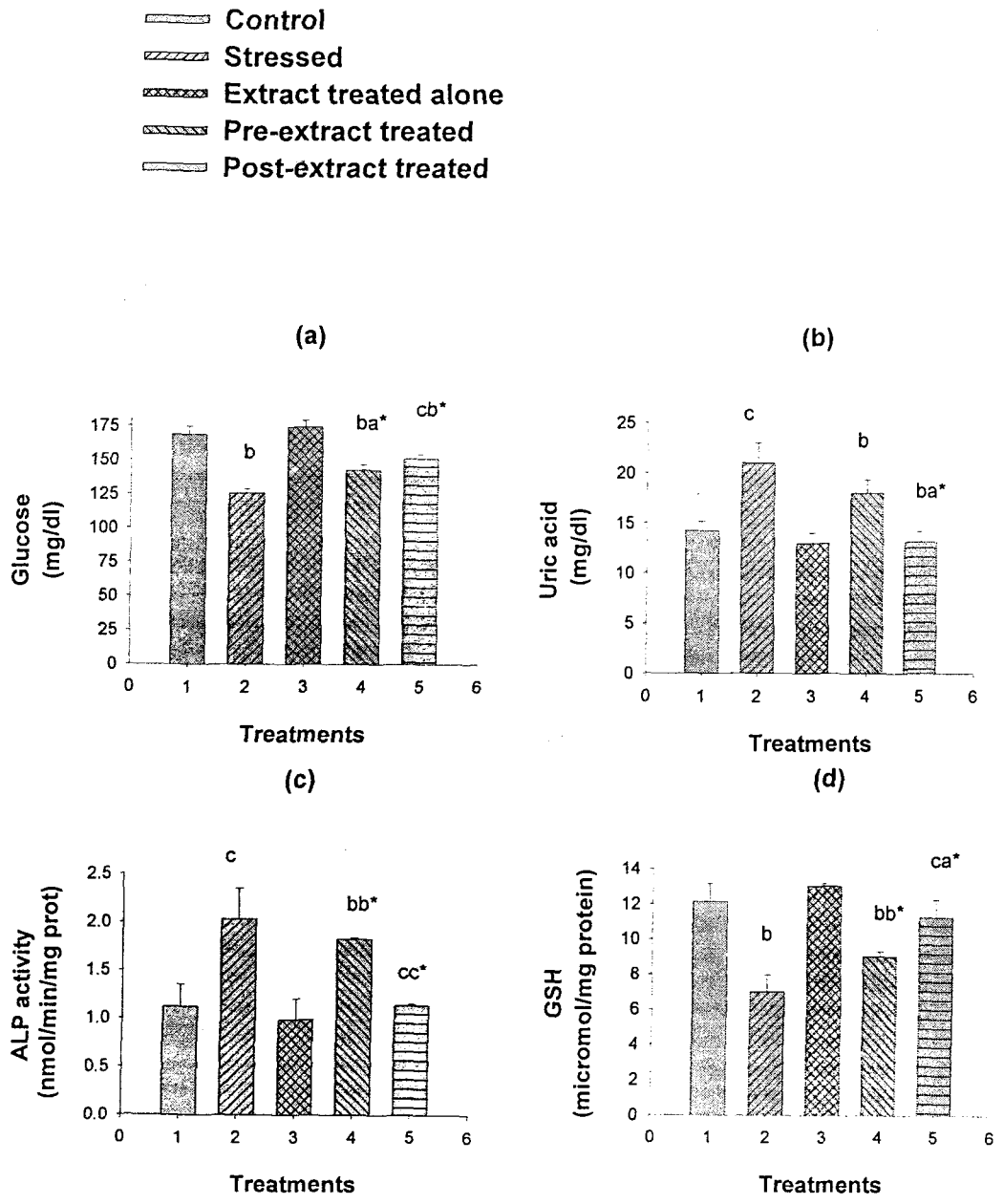
a = p<0.02, b = p<0.05 and c = p<0.001 shows p value as compared to controls where as a* = p<0.02, b* = p<0.05 and c* = p<0.001 shows p values as compared to stressed rats.

Fig- 4. Effect of crude extract of *S. nigrum* on immobilization stress induced changes in Liver tissue levels of SOD Catalase, GST and MDA.



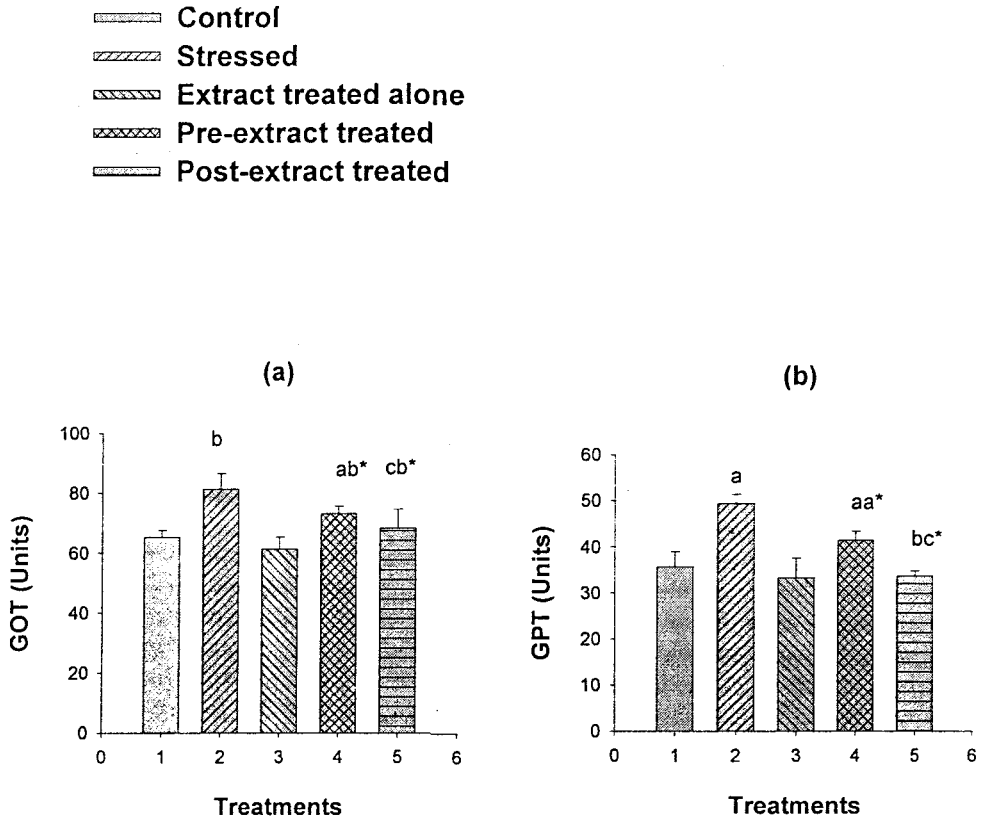
a = p<0.02, b = p<0.05 and c = p<0.001 shows p value as compared to controls where as a* = p<0.02, b* = p<0.05 and c* = p<0.001 shows p values as compared to stressed rats.

Fig- 5. Effect of crude extract of *S. nigrum* on immobilization stress induced changes in Liver tissue levels of Glucose, Uric acid, ALP and GSH.



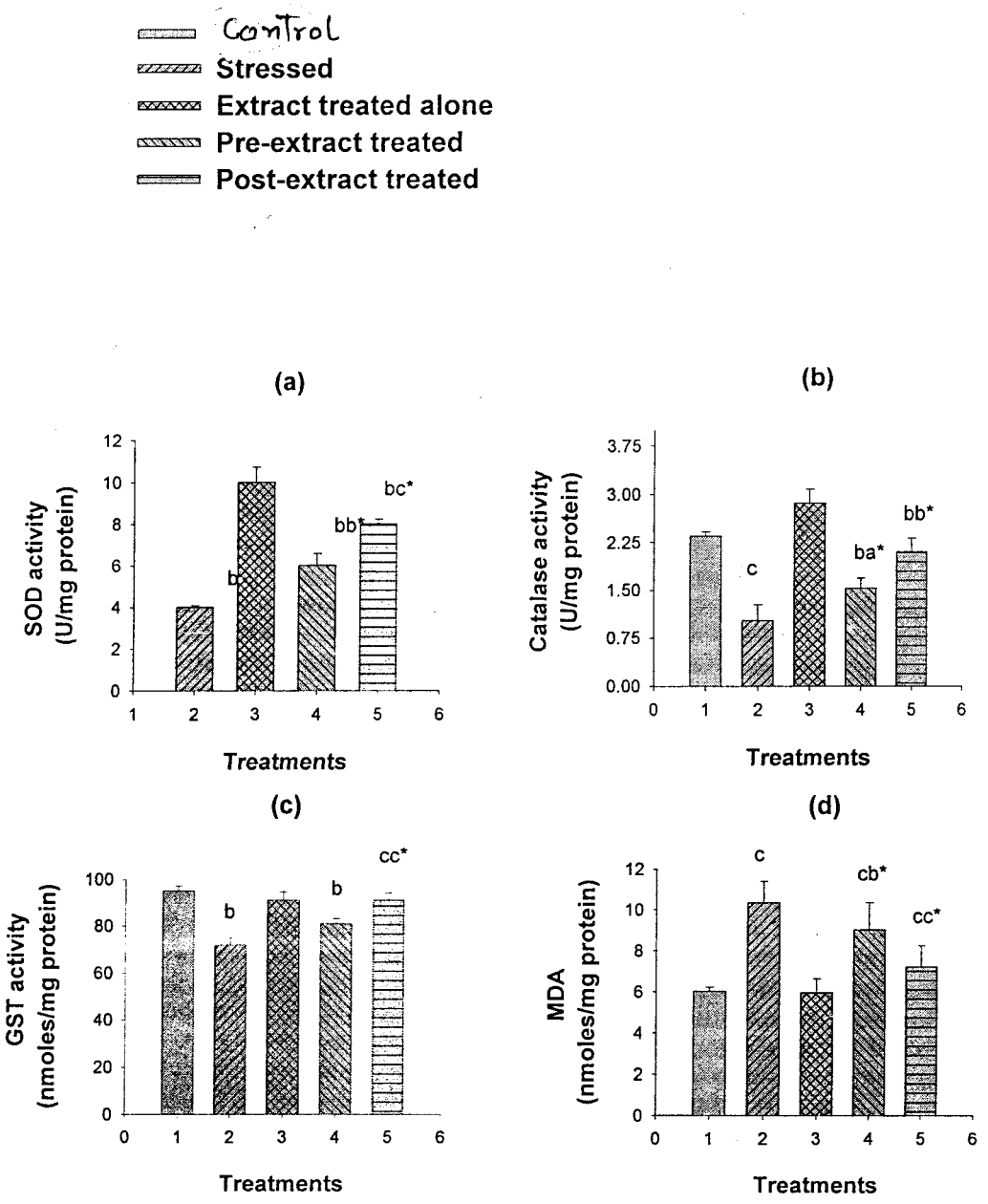
a = p<0.02, b = p<0.05 and c = p<0.001 shows p value as compared to controls where as a* = p<0.02, b* = p<0.05 and c* = p<0.001 shows p values as compared to stressed rats.

Fig- 6. Effect of crude extract of *S. nigrum* on immobilization stress Induced changes in Liver levels of GOT and GPT.



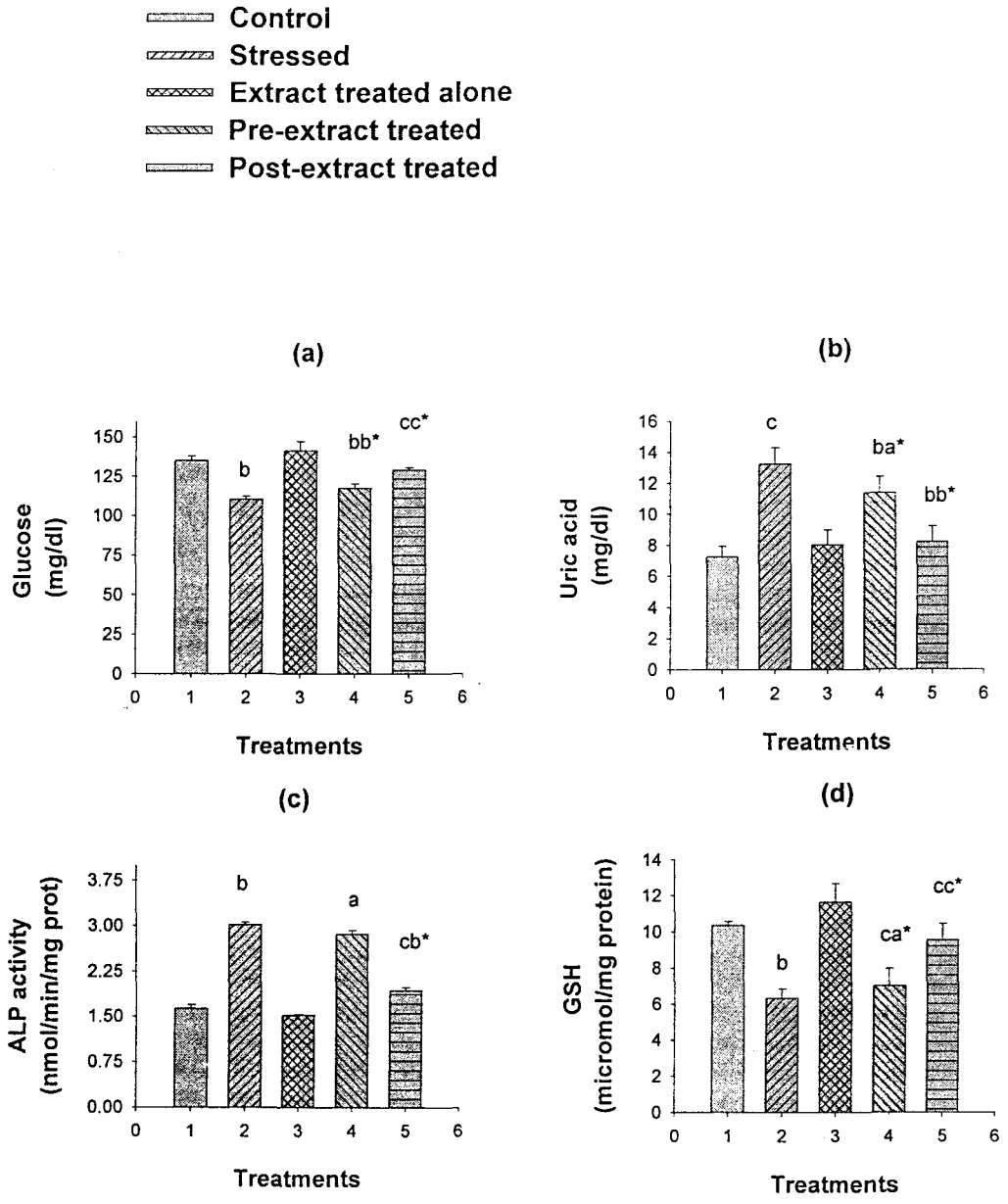
a = p<0.02, b = p<0.05 and c = p<0.001 shows p value as compared to controls where as a* = p<0.02, b* = p<0.05 and c* = p<0.001 shows p values as compared to stressed rats.

Fig- 7. Effect of crude extract of *S. nigrum* on immobilization stress changes in Heart levels of SOD, GST, Catalase and MDA.



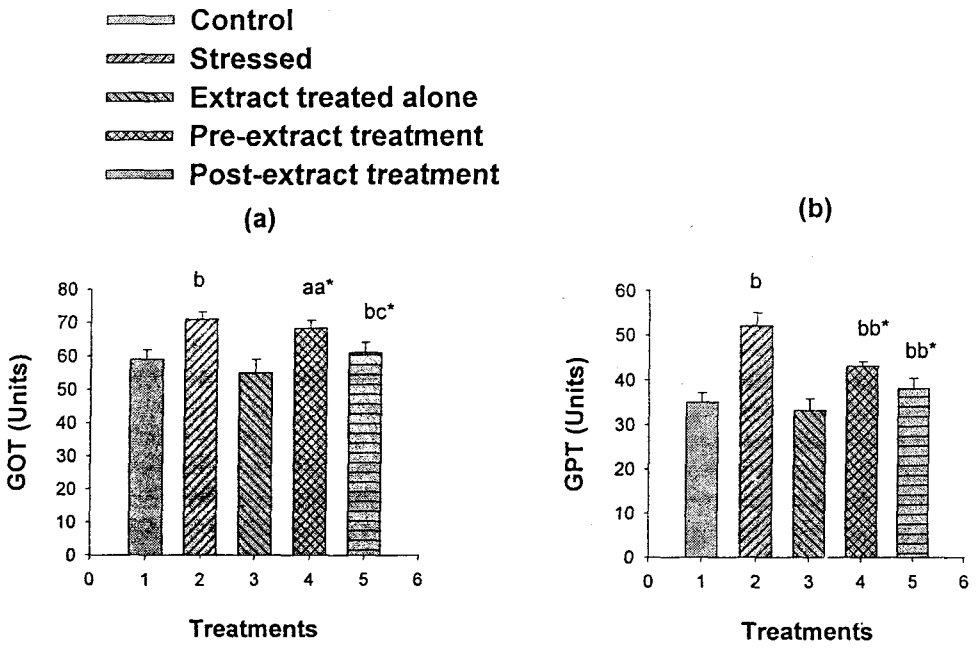
a = p<0.02, b = p<0.05 and c = p<0.001 shows p value as compared to controls where as a* = p<0.02, b* = p<0.05 and c* = p<0.001 shows p values as compared to stressed rats.

Fig-8. **Effect of crude extract of *S. nigrum* on immobilization stress induced changes in Heart levels of Glucose, uric acid GSH and ALP.**



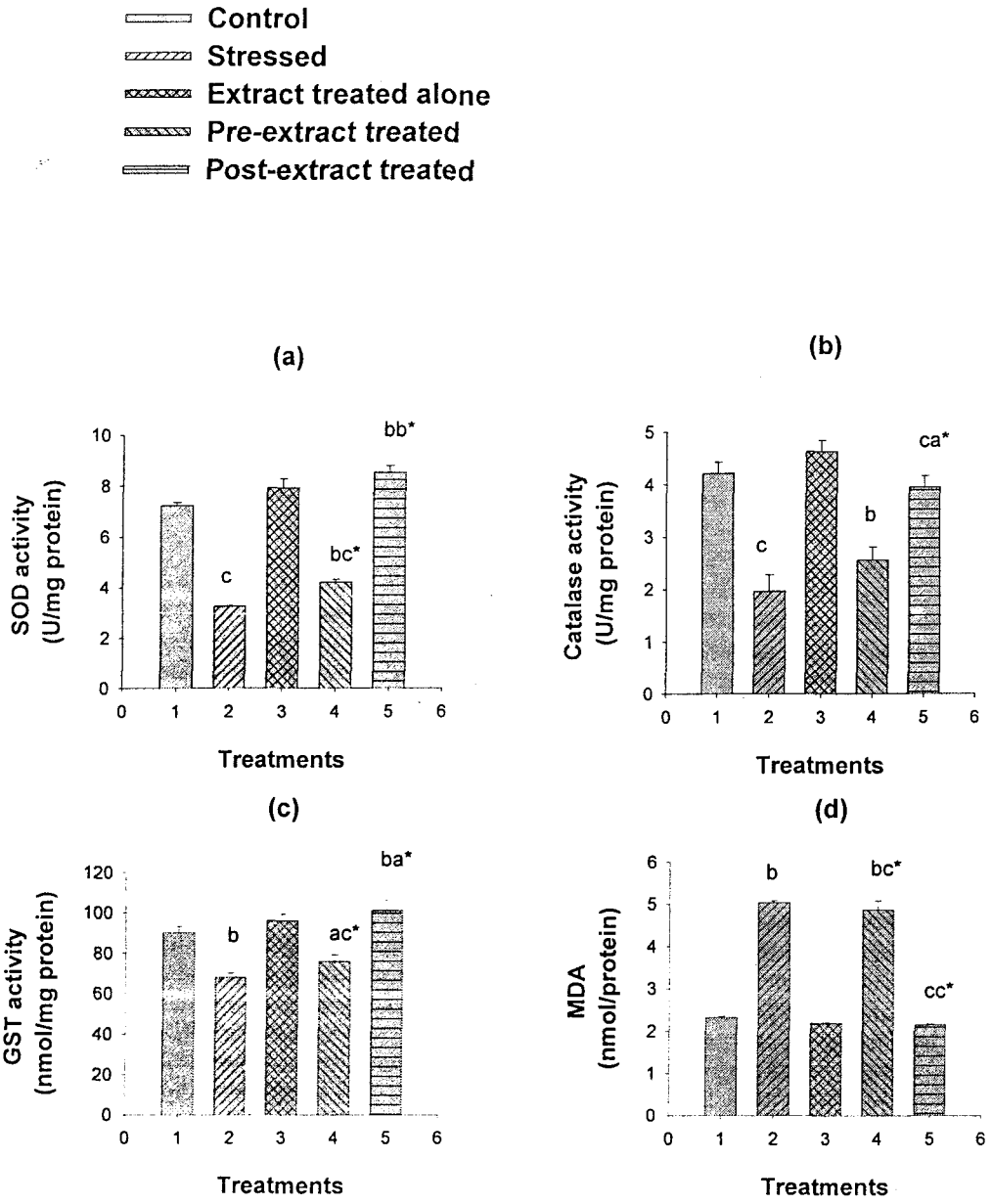
a = p<0.02, b = p<0.05 and c = p<0.001 shows p value as compared to controls where as a* = p<0.02, b* = p<0.05 and c* = p<0.001 shows p values as compared to stressed rats.

Fig- 9. Effect of crude extract of *S. nigrum* on immobilization stress induced changes in Heart levels of GOT and GPT.



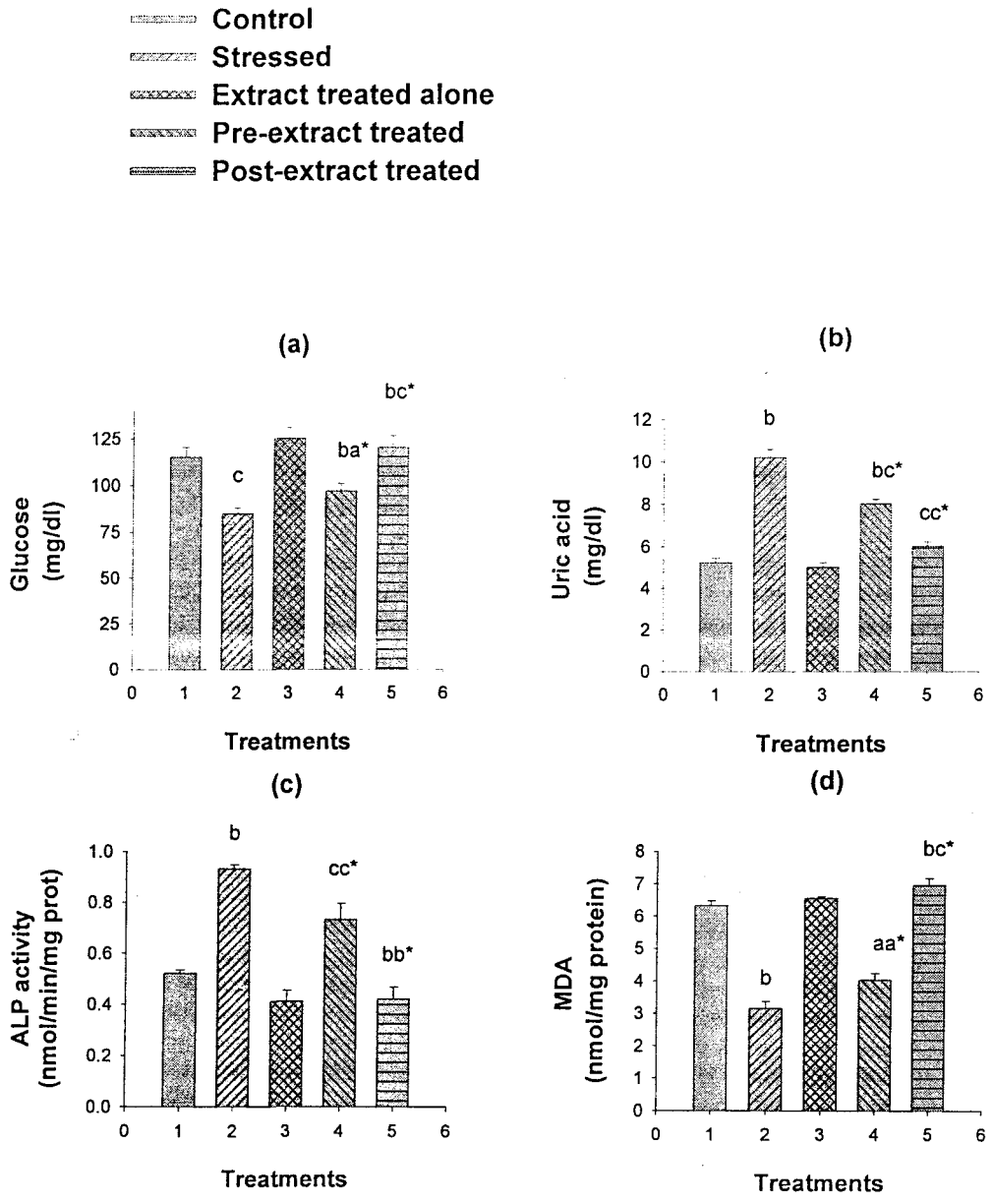
a = p<0.02, b = p<0.05 and c = p<0.001 shows p value as compared to controls where as a* = p<0.02, b* = p<0.05 and c* = p<0.001 shows p values as compared to stressed rats.

Fig- 10. Effect of crude extract of *S. nigrum* on immobilization stress induced changes in Spleen tissue levels of SOD, GST catalase and MDA.



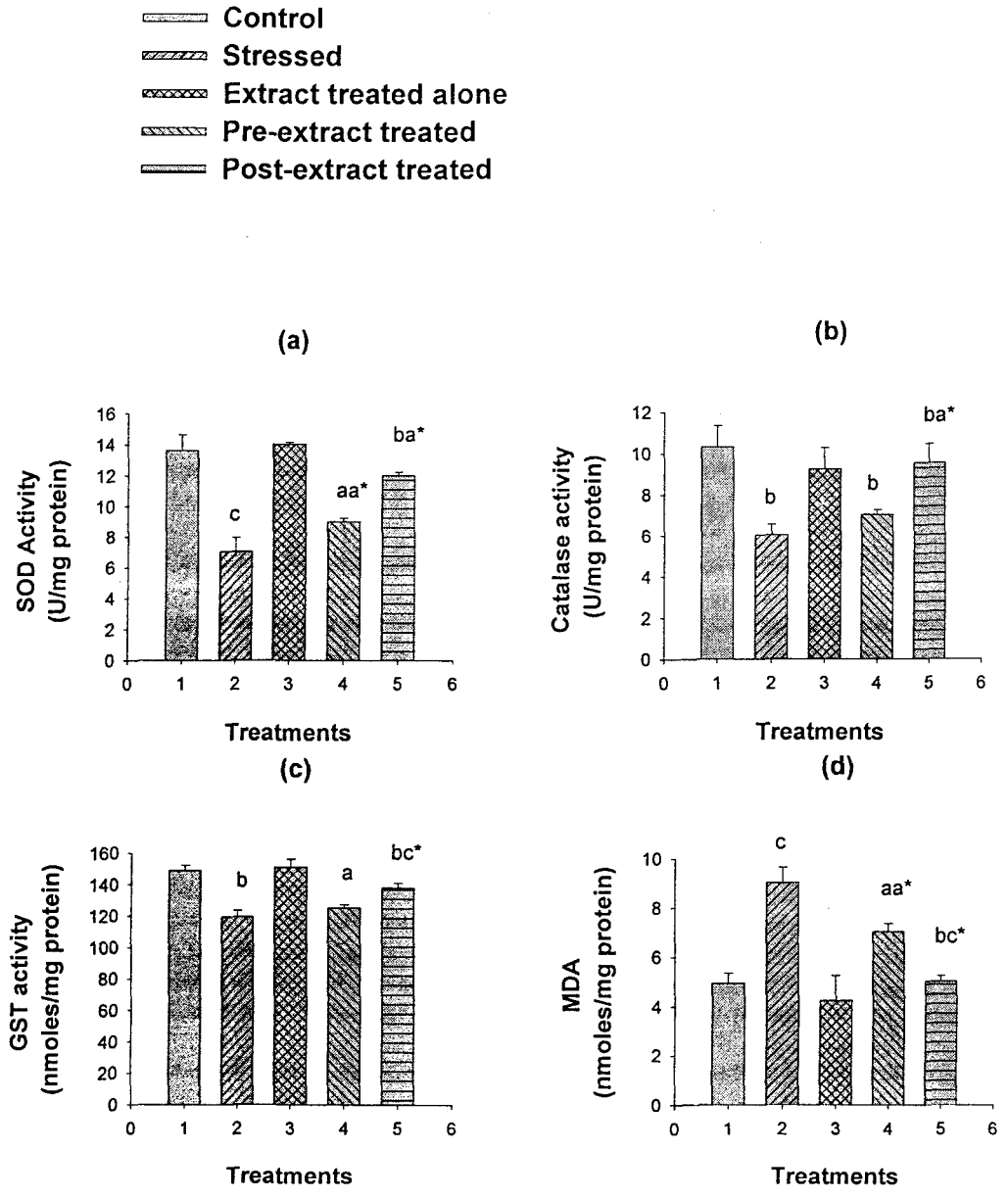
a = p<0.02, b = p<0.05 and c = p<0.001 shows p value as compared to controls where as a* = p<0.02, b* = p<0.05 and c* = p<0.001 shows p values as compared to stressed rats.

Fig- 11. Effect of crude extract of *S. nigrum* on immobilization stress induced changes in Spleen tissue levels of Glucose, Uric acid, GSH and ALP.



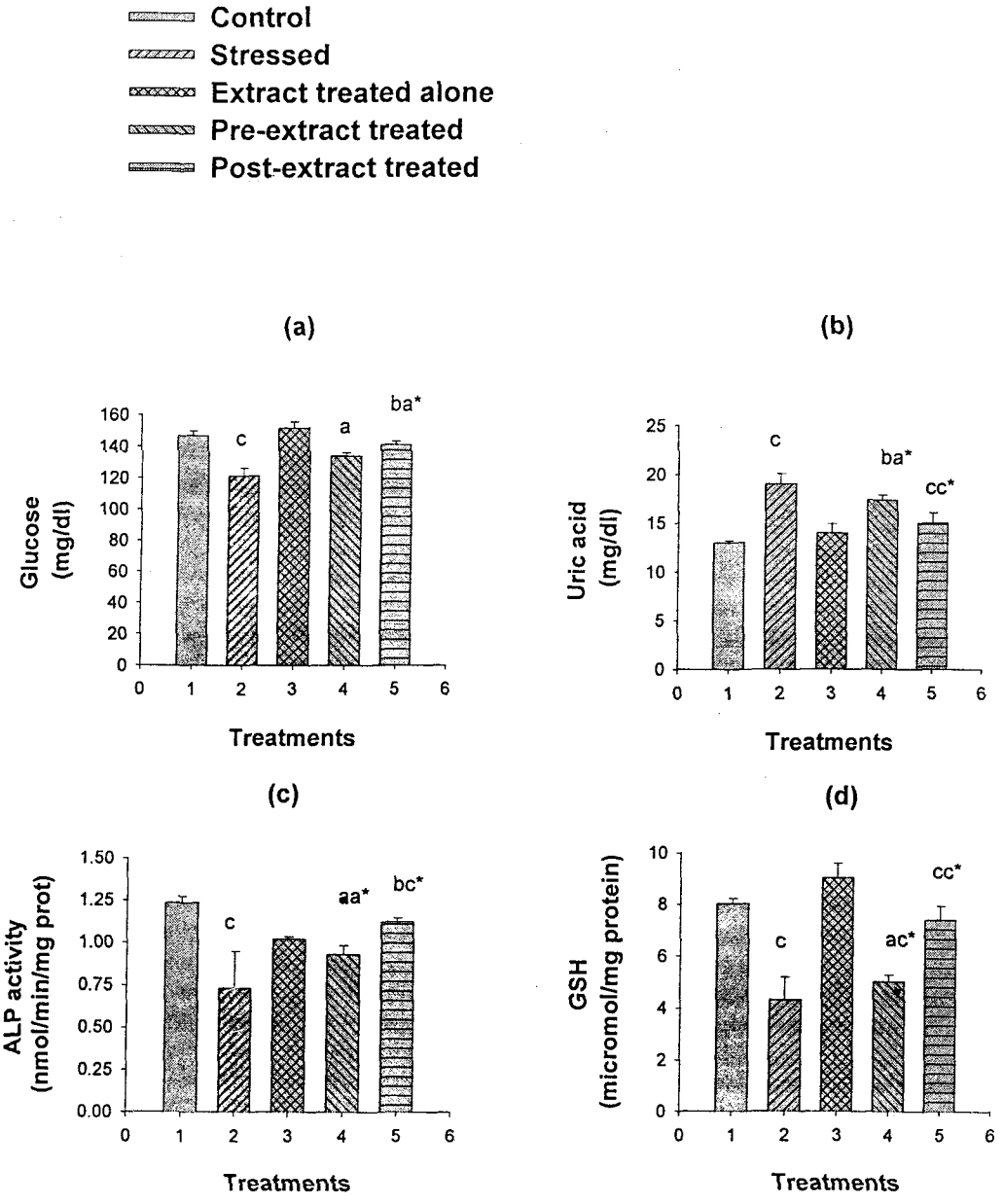
a = p<0.02, b = p<0.05 and c = p<0.001 shows p value as compared to controls where as a* = p<0.02, b* = p<0.05 and c* = p<0.001 shows p values as compared to stressed rats.

Fig- 12. Effect of crude extract of *S. nigrum* on immobilization stress induced changes in Kidney tissue levels of SOD, GST, Catalase and MDA.



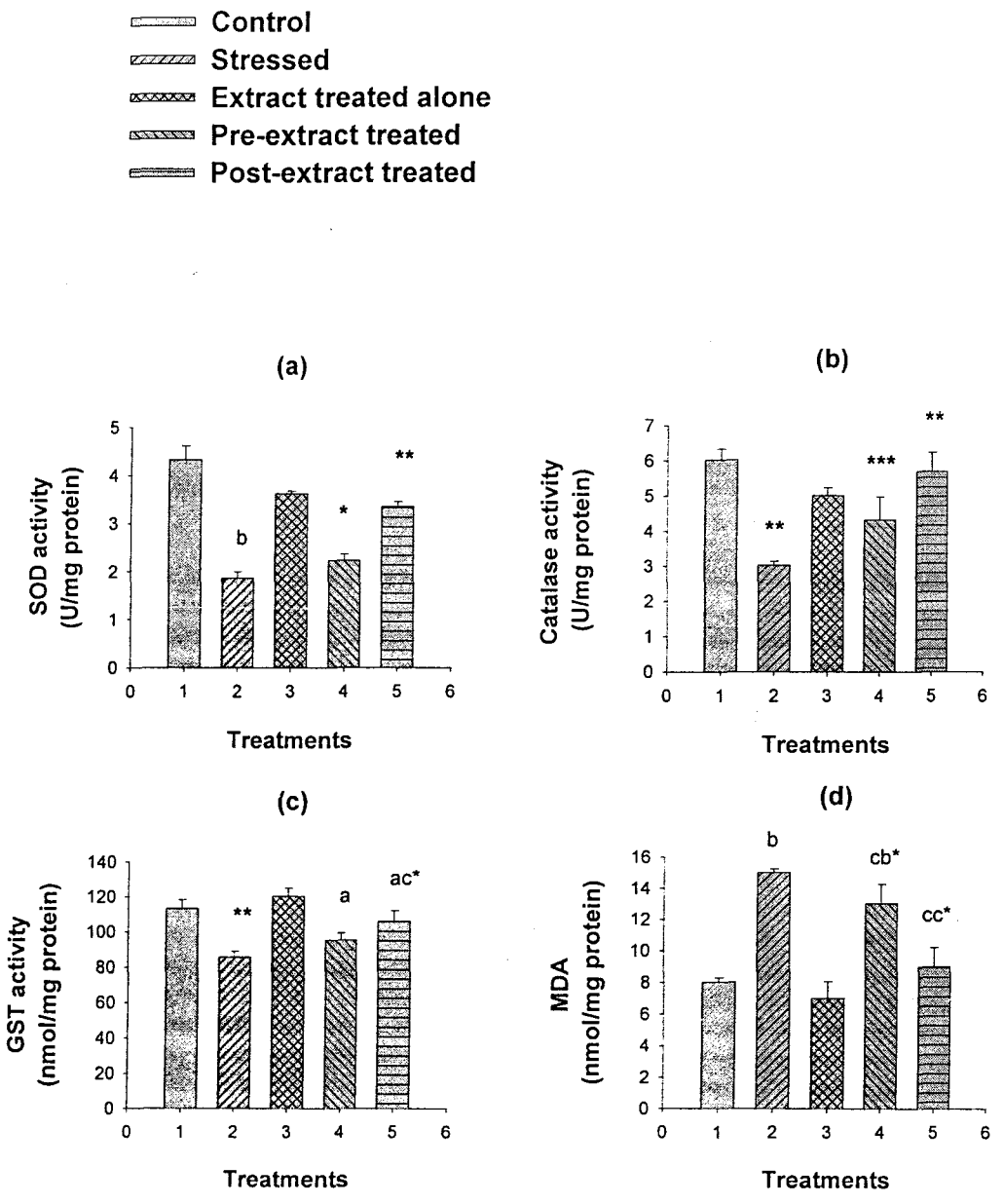
a = p<0.02, b = p<0.05 and c = p<0.001 shows p value as compared to controls where as a* = p<0.02, b* = p<0.05 and c* = p<0.001 shows p values as compared to stressed rats.

Fig- 13. Effect of crude extract of *S. nigrum* on immobilization stress induced changes in Kidney tissue levels of Glucose, uric acid, GSH and ALP.



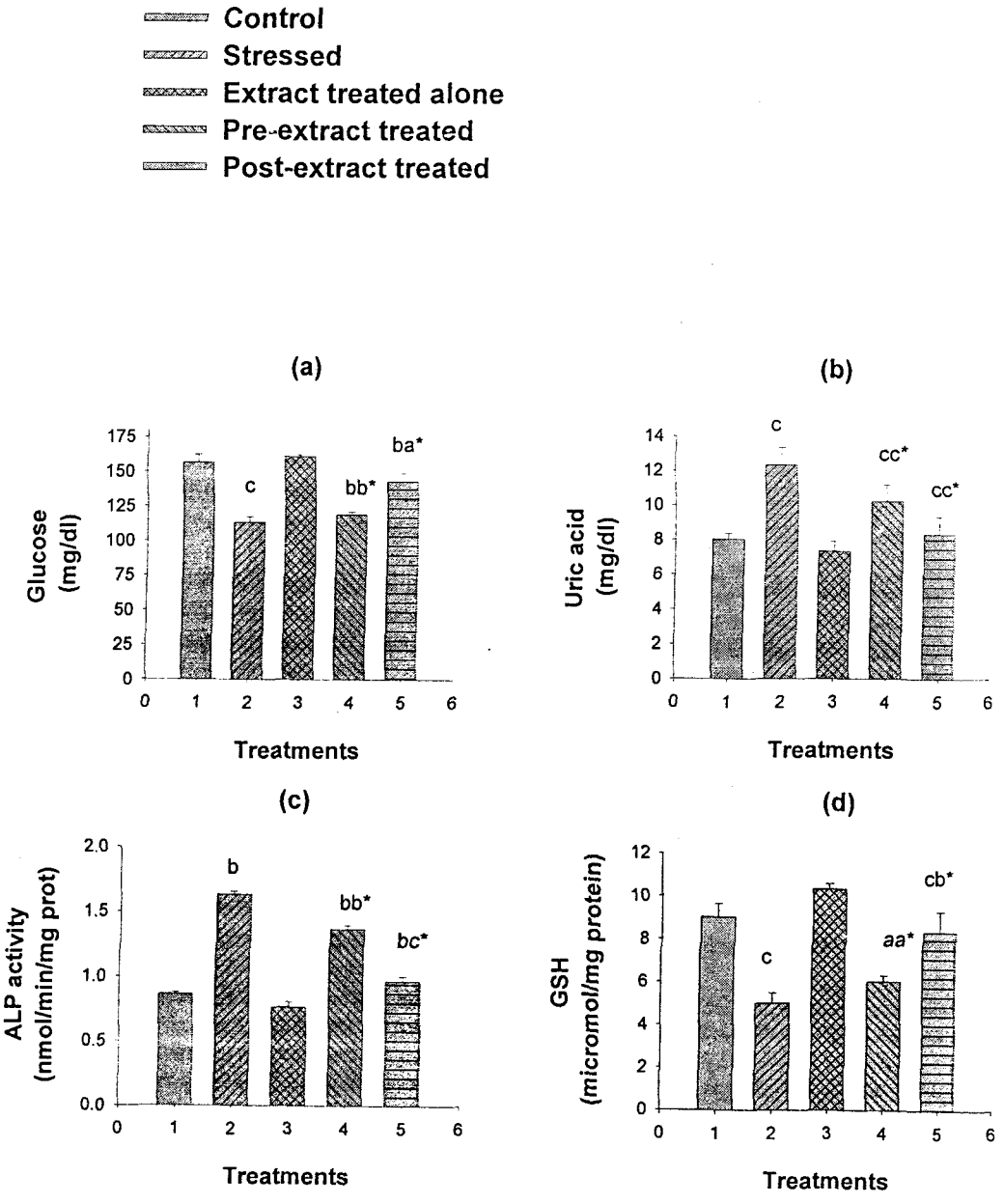
a = p<0.02, *b* = p<0.05 *and c* = p<0.001 shows p value as compared to controls where as a* = p<0.02, b* = p<0.05 and c* = p<0.001 shows p values as compared to stressed rats.

Fig- 14 Effect of crude extract of *S. nigrum* on immobilization stress induced changes in Brain tissue levels of SOD, Catalase, GST and MDA



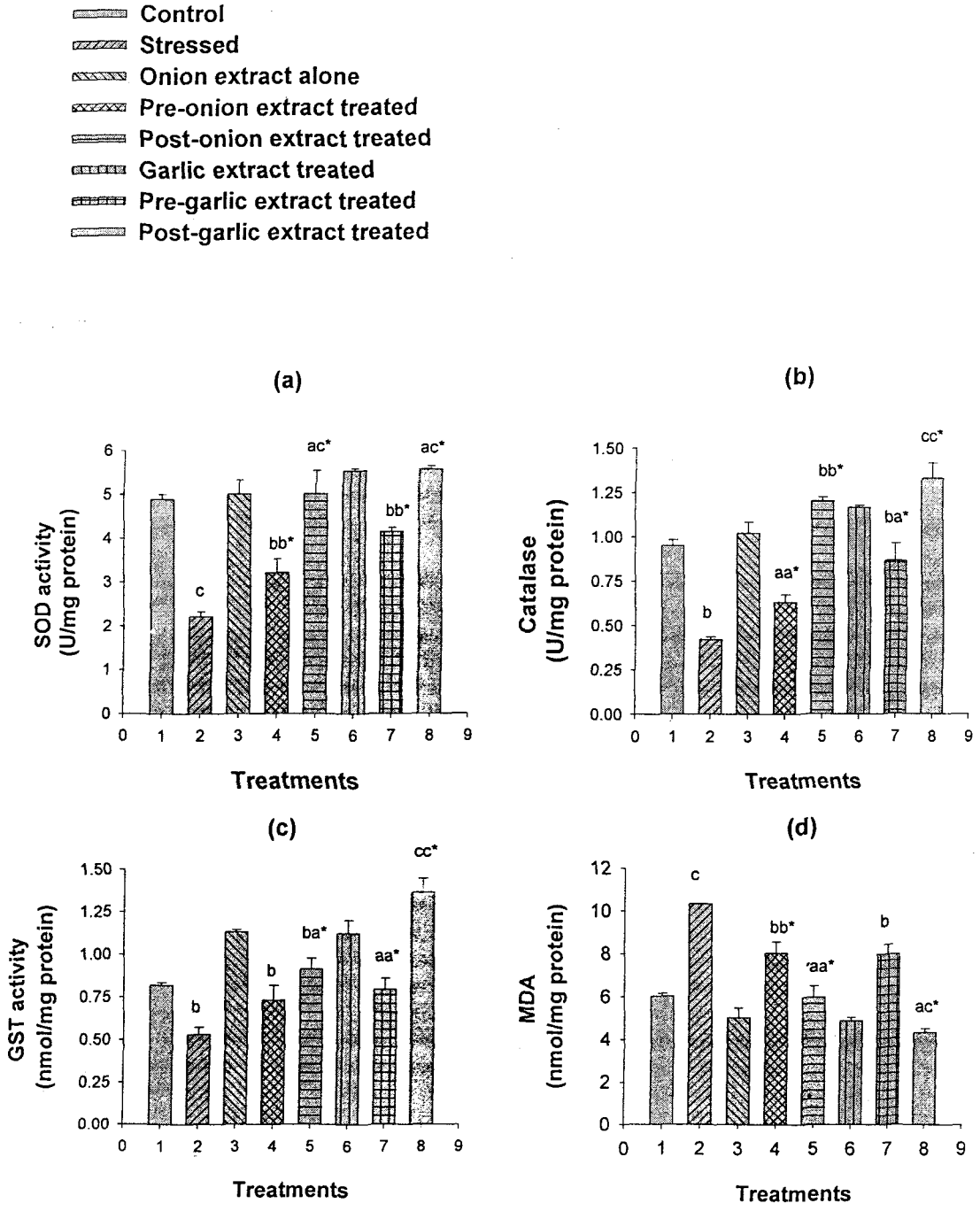
a = p<0.02, b = p<0.05 and c = p<0.001 shows p value as compared to controls where as a* = p<0.02, b* = p<0.05 and c* = p<0.001 shows p values as compared to stressed rats.

Fig- 15. Effect of crude extract of *S. nigrum* on immobilization stress induced changes in Brain tissue levels of Glucose Uric acid, ALP and GSH,



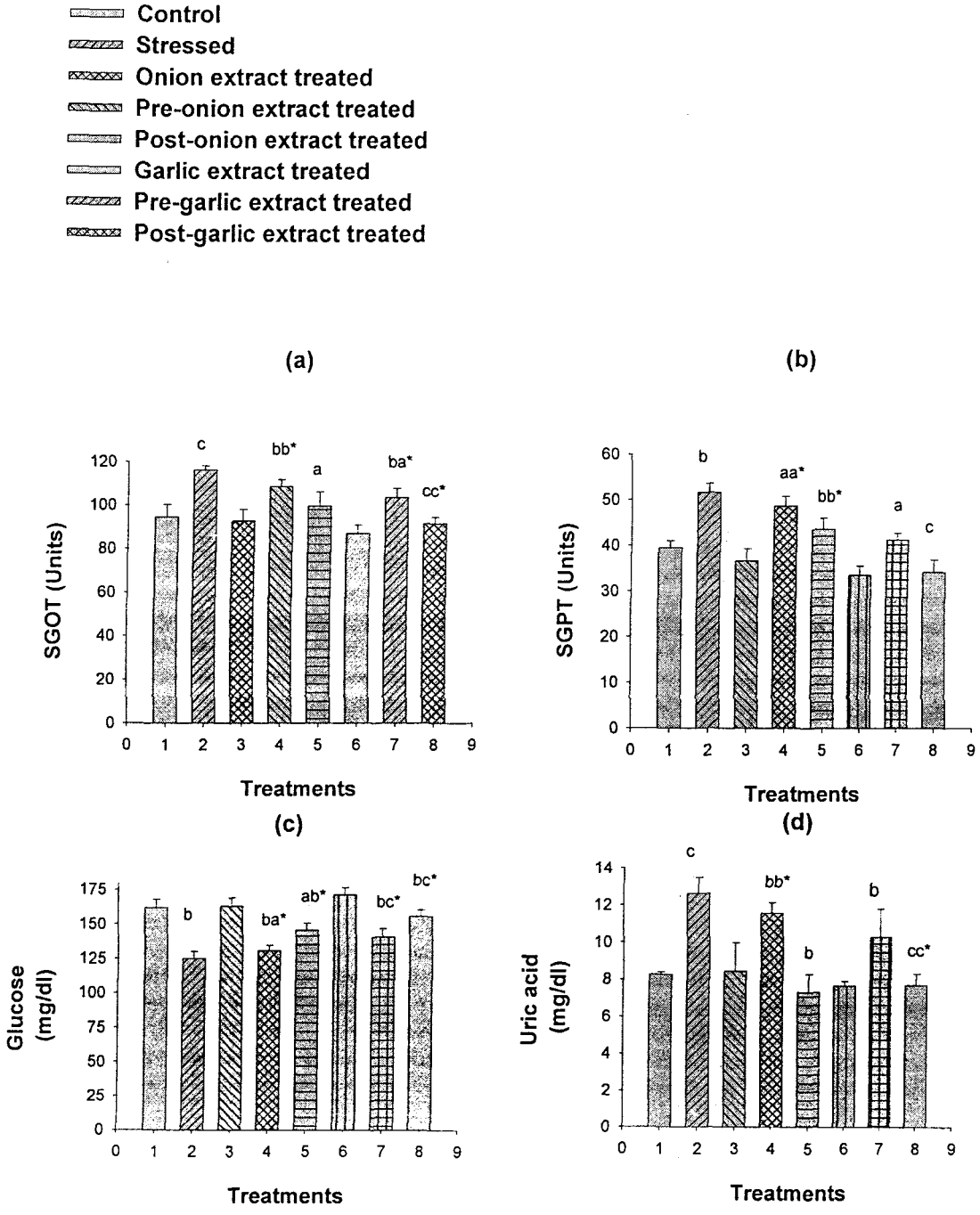
a = p<0.02, b = p<0.05 and c = p<0.001 shows p value as compared to controls where as a* = p<0.02, b* = p<0.05 and c* = p<0.001 shows p values as compared to stressed rats.

Fig- 16. Effect of garlic and onion extracts on immobilization stress induced changes in circulating levels of SOD, Catalase, GST and MDA



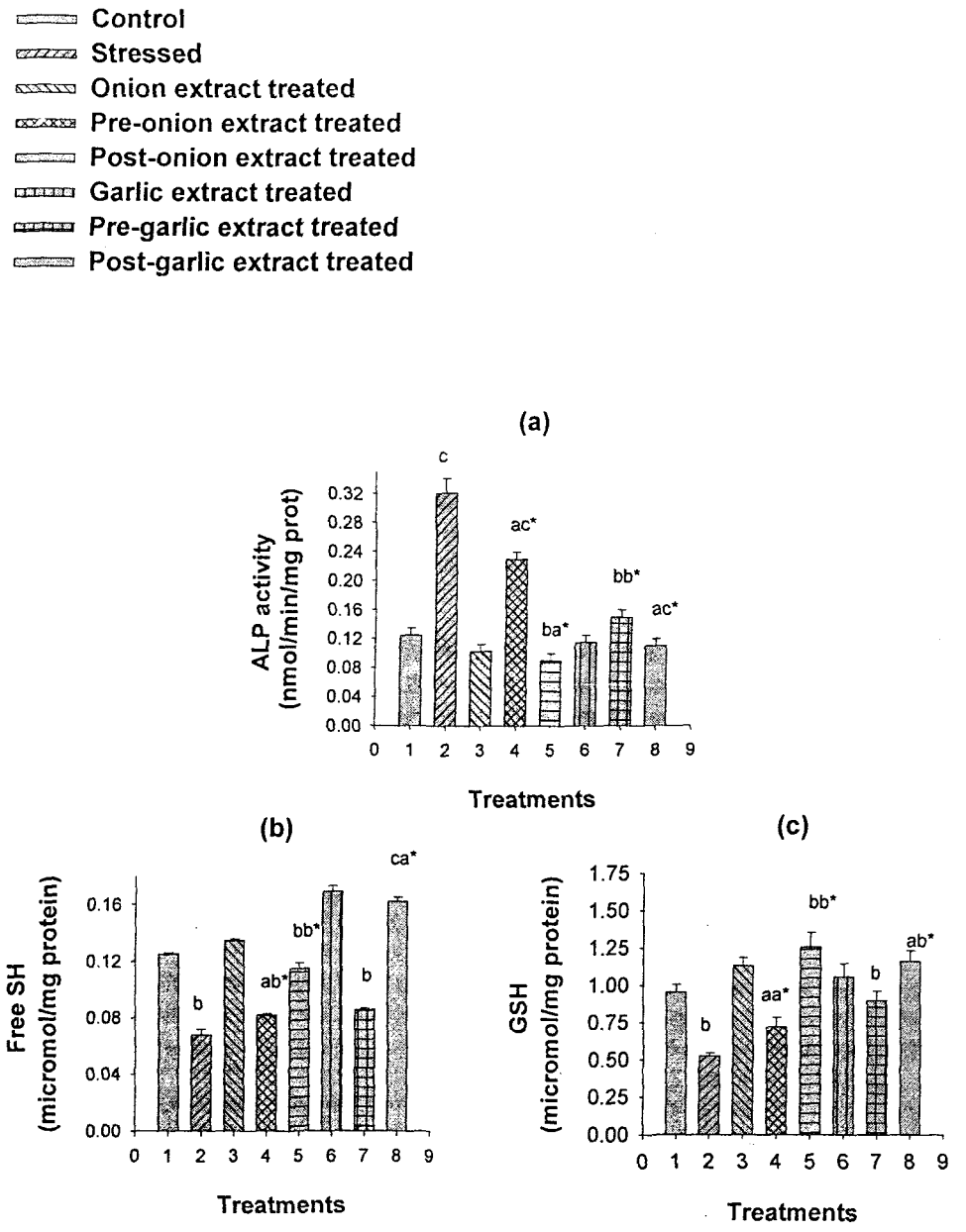
a = p<0.02, b = p<0.05 and c = p<0.001 shows p value as compared to controls where as a* = p<0.02, b* = p<0.05 and c* = p<0.001 shows p values as compared to stressed rats.

Fig- 17. Effect of garlic and onion extracts on immobilization stress induced changes in circulating levels of SGOT, SGPT, Glucose and uric acid.



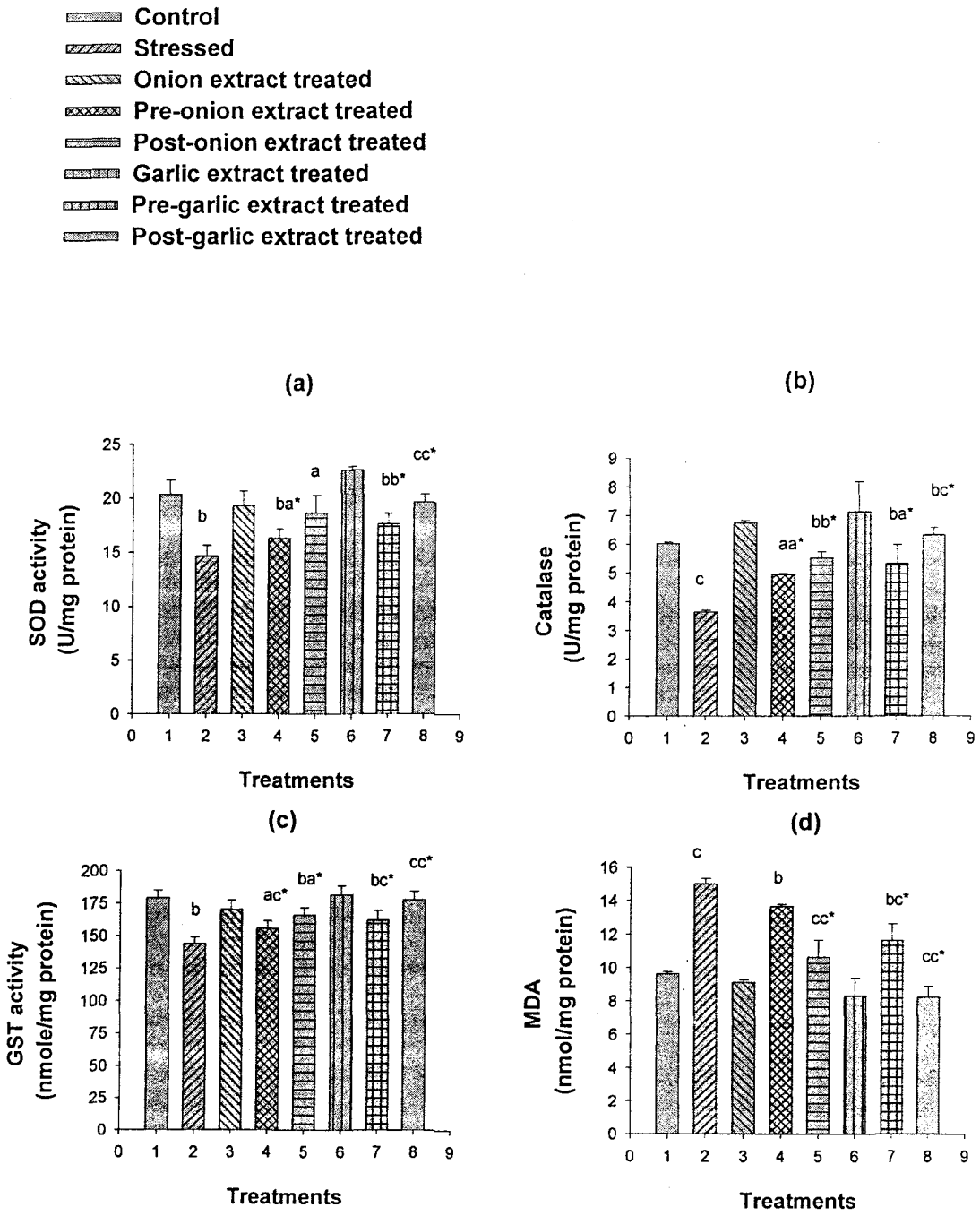
a = p<0.02, b = p<0.05 and c = p<0.001 shows p value as compared to controls where as a* = p<0.02, b* = p<0.05 and c* = p<0.001 shows p values as compared to stressed rats.

Fig- 18. Effect of garlic and onion extracts on immobilization stress induced changes in circulating levels of ALP, Total SH and Free SH groups



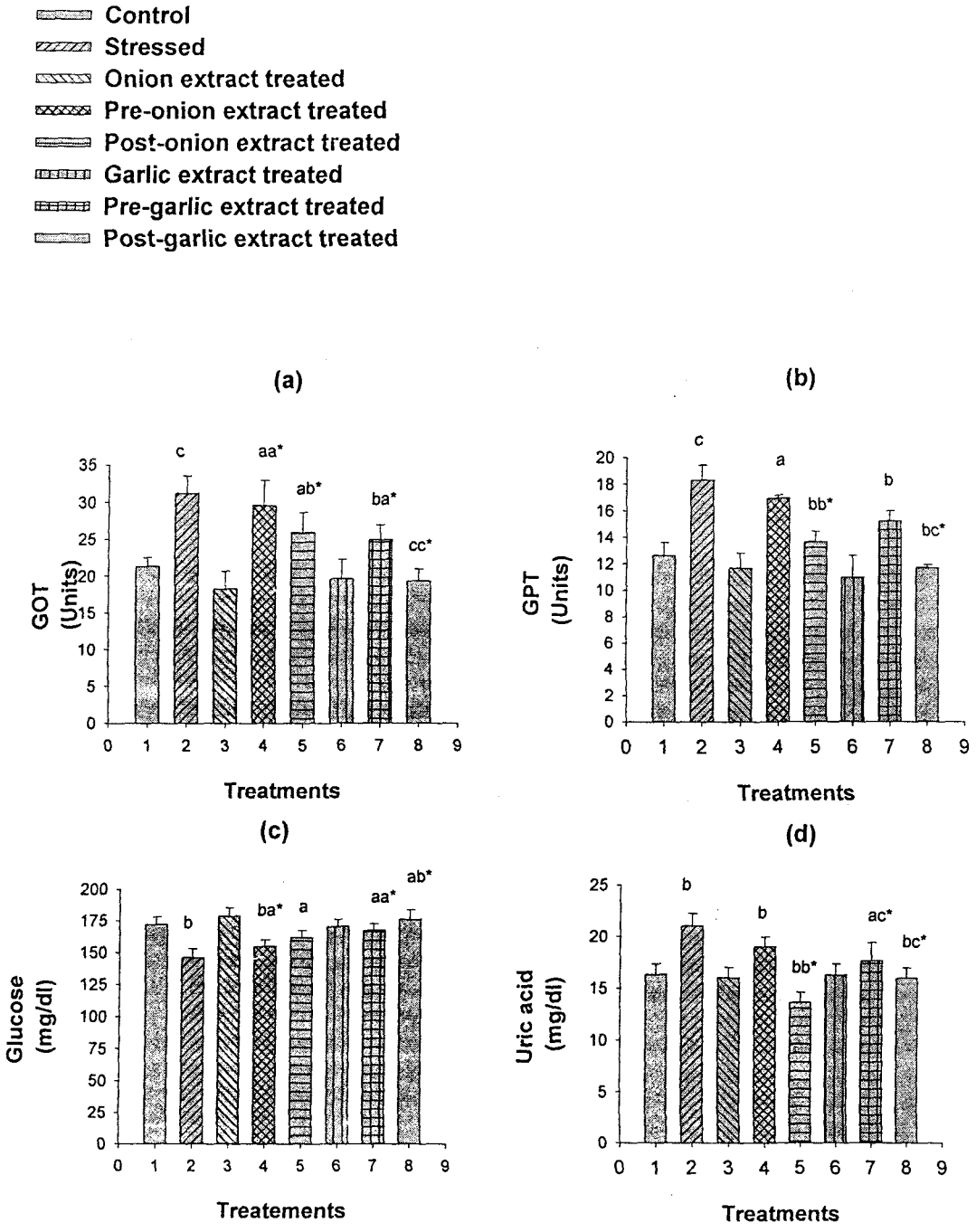
a = p<0.02, b = p<0.05 and c = p<0.001 shows p value as compared to controls where as a* = p<0.02, b* = p<0.05 and c* = p<0.001 shows p values as compared to stressed rats.

Fig- 19. Effect of garlic and onion extracts on immobilization stress induced changes in Liver levels of SOD, Catalase, GST and MDA



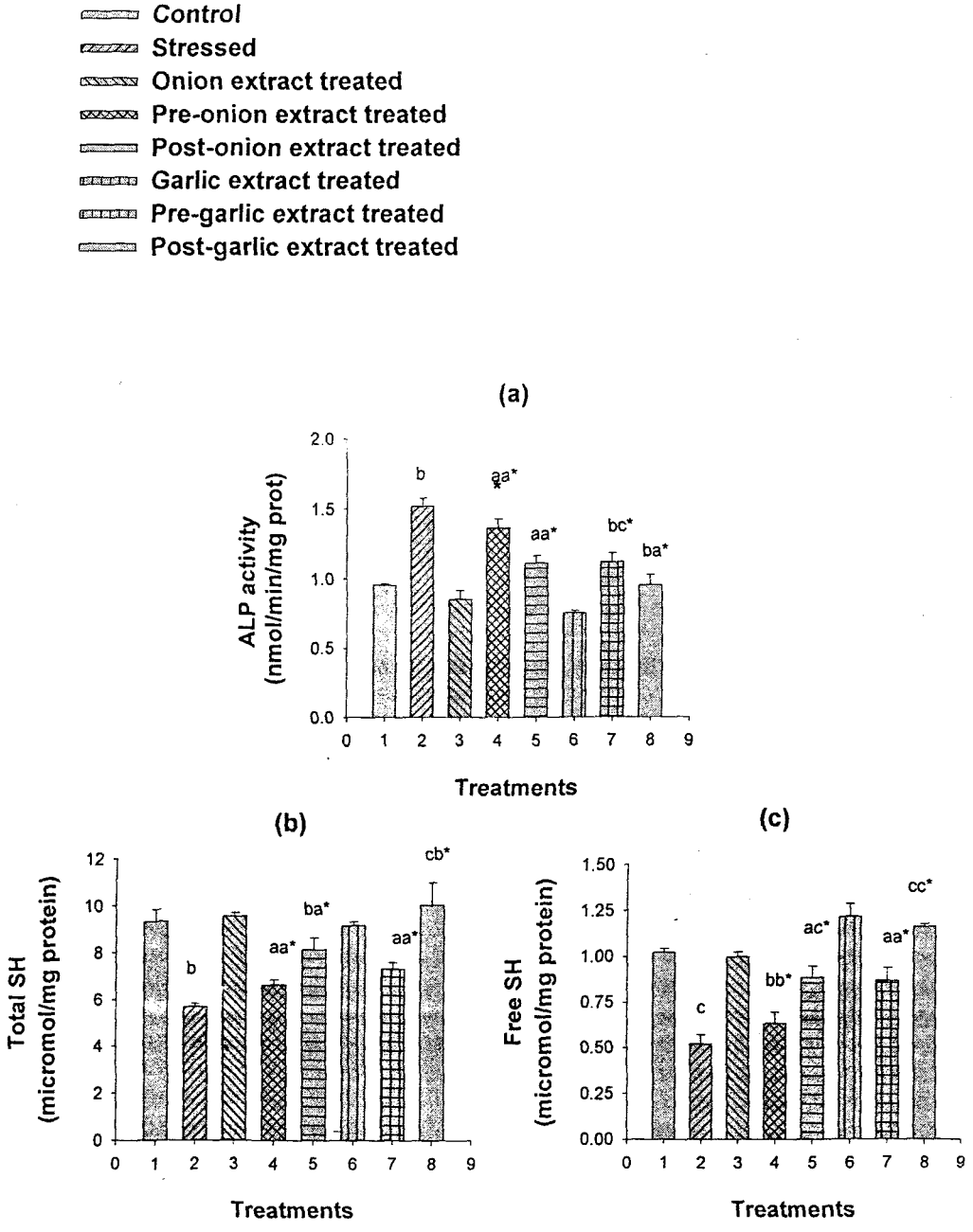
a = p<0.02, b = p<0.05 and c = p<0.001 shows p value as compared to controls where as a* = p<0.02, b* = p<0.05 and c* = p<0.001 shows p values as compared to stressed rats.

Fig- 20. Effect of garlic and onion extracts on immobilization stress induced changes in Liver levels of GOT, GPT, Glucose and uric acid



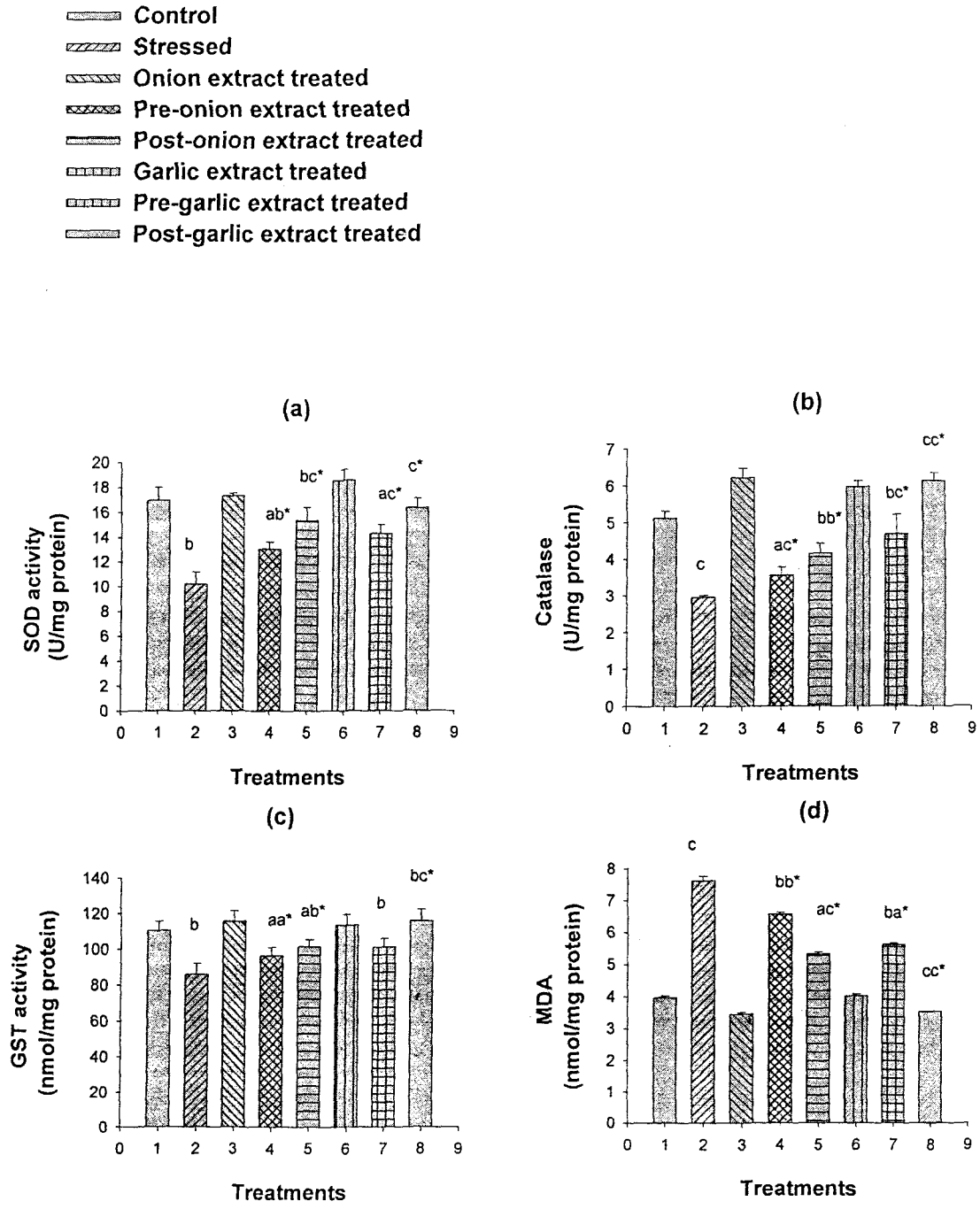
a = p<0.02, b = p<0.05 and c = p<0.001 shows p value as compared to controls where as a* = p<0.02, b* = p<0.05 and c* = p<0.001 shows p values as compared to stressed rats.

Fig- 21. Effect of garlic and onion extracts on immobilization stress induced changes in Liver levels of ALP total and free SH groups.



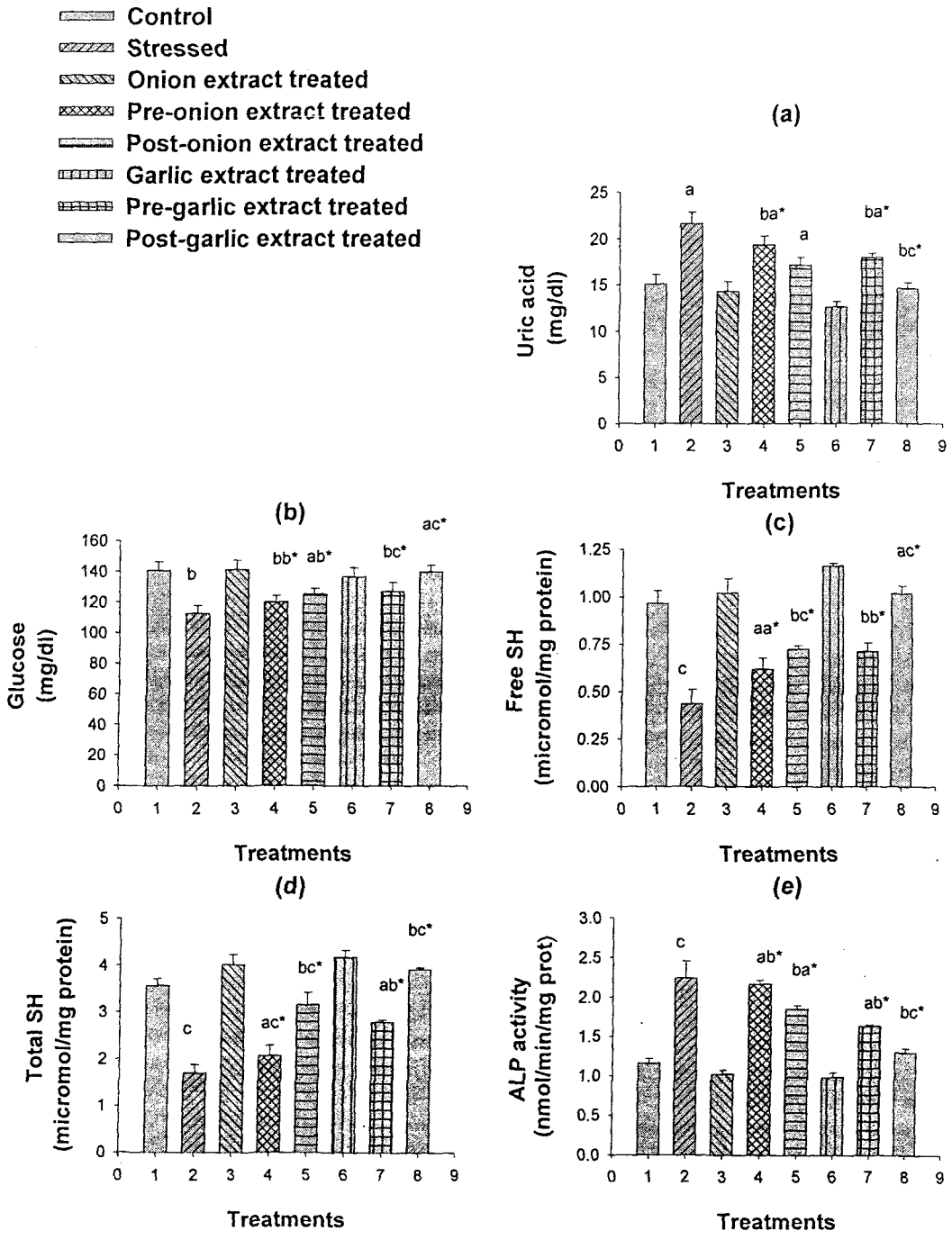
a = p<0.02, b = p<0.05 and c = p<0.001 shows p value as compared to controls where as a* = p<0.02, b* = p<0.05 and c* = p<0.001 shows p values as compared to stressed rats.

Fig- 22. Effect of garlic and onion extracts on immobilization stress induced changes in Spleen tissue levels of SOD, Catalase, GST and MDA



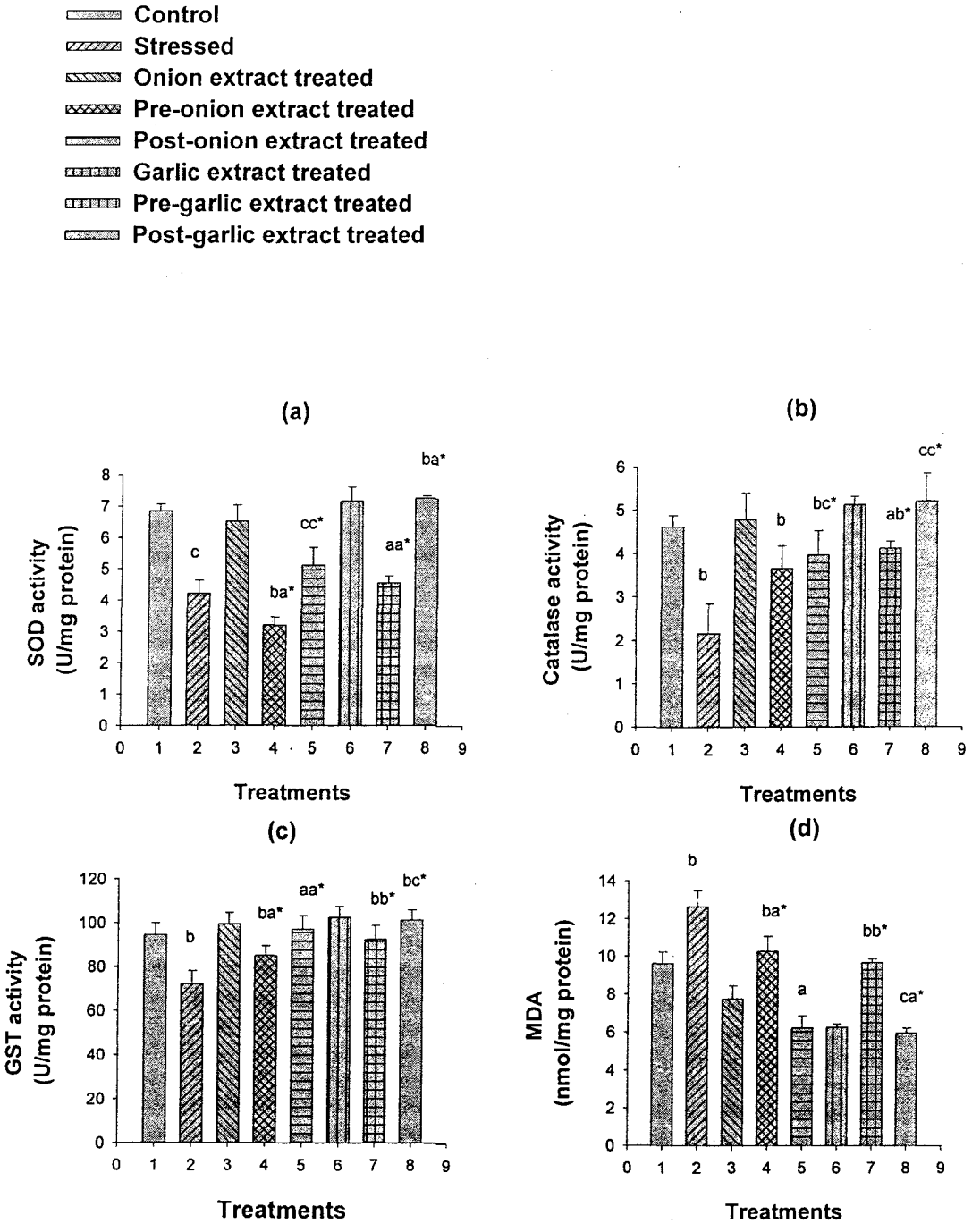
a = p<0.02, b = p<0.05 and c = p<0.001 shows p value as compared to controls where as a* = p<0.02, b* = p<0.05 and c* = p<0.001 shows p values as compared to stressed rats.

Fig- 23. Effect of garlic and onion extracts on immobilization stress induced changes in Spleen tissue levels of Glucose, uric acid, ALP, total and free SH groups



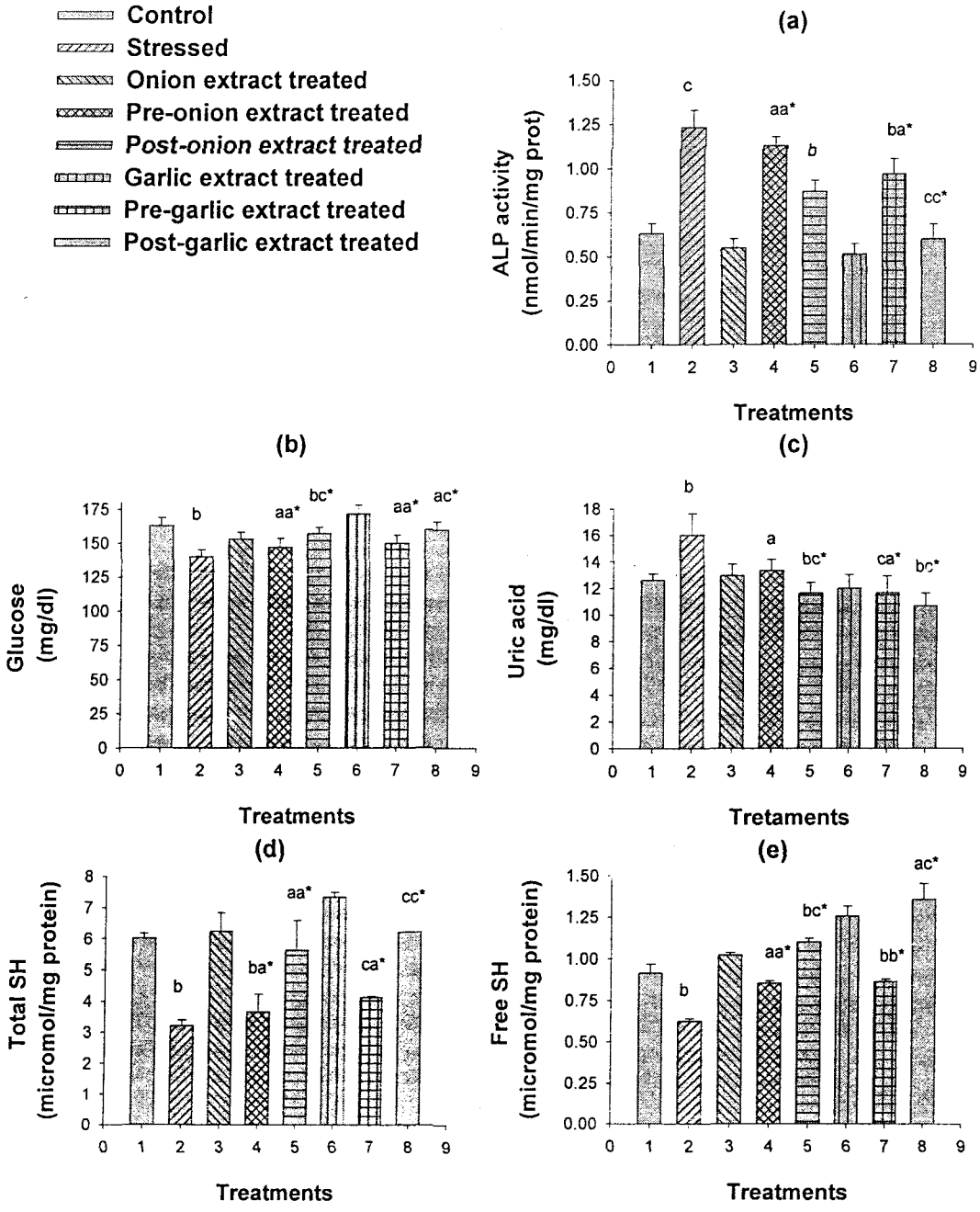
a = p<0.02, b = p<0.05 and c = p<0.001 shows p value as compared to controls where as a* = p<0.02, b* = p<0.05 and c* = p<0.001 shows p values as compared to stressed rats.

Fig- 24. Effect of garlic and onion extracts on immobilization stress induced changes in Brain levels of SOD, Catalase, GST and MDA



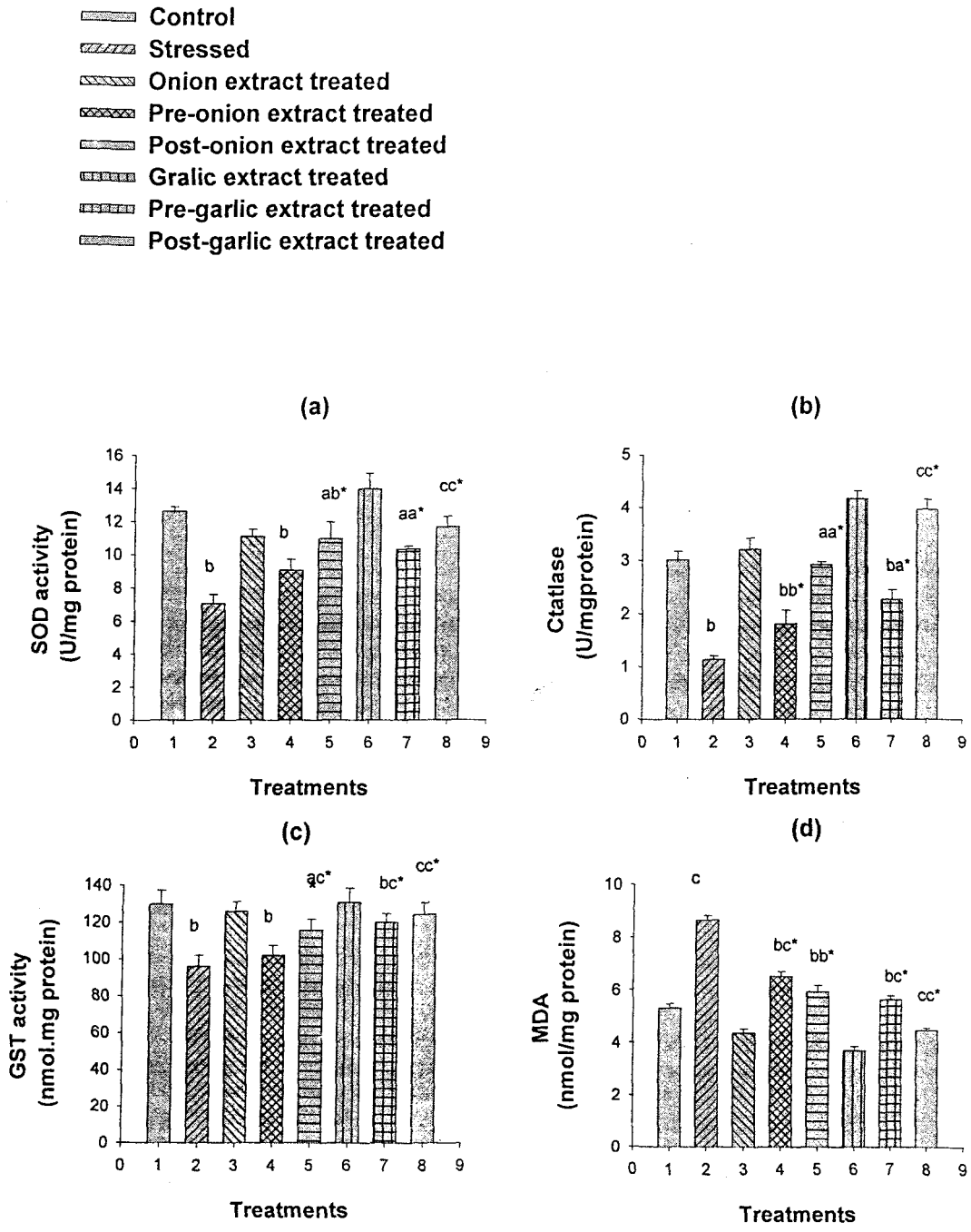
a = p<0.02, b = p<0.05 and c = p<0.001 shows p value as compared to controls where as a* = p<0.02, b* = p<0.05 and c* = p<0.001 shows p values as compared to stressed rats.

Fig- 25. Effect of garlic and onion extracts on immobilization stress induced changes in Brain levels of ALP, Glucose, Uric acid, Total SH and Free SH groups.



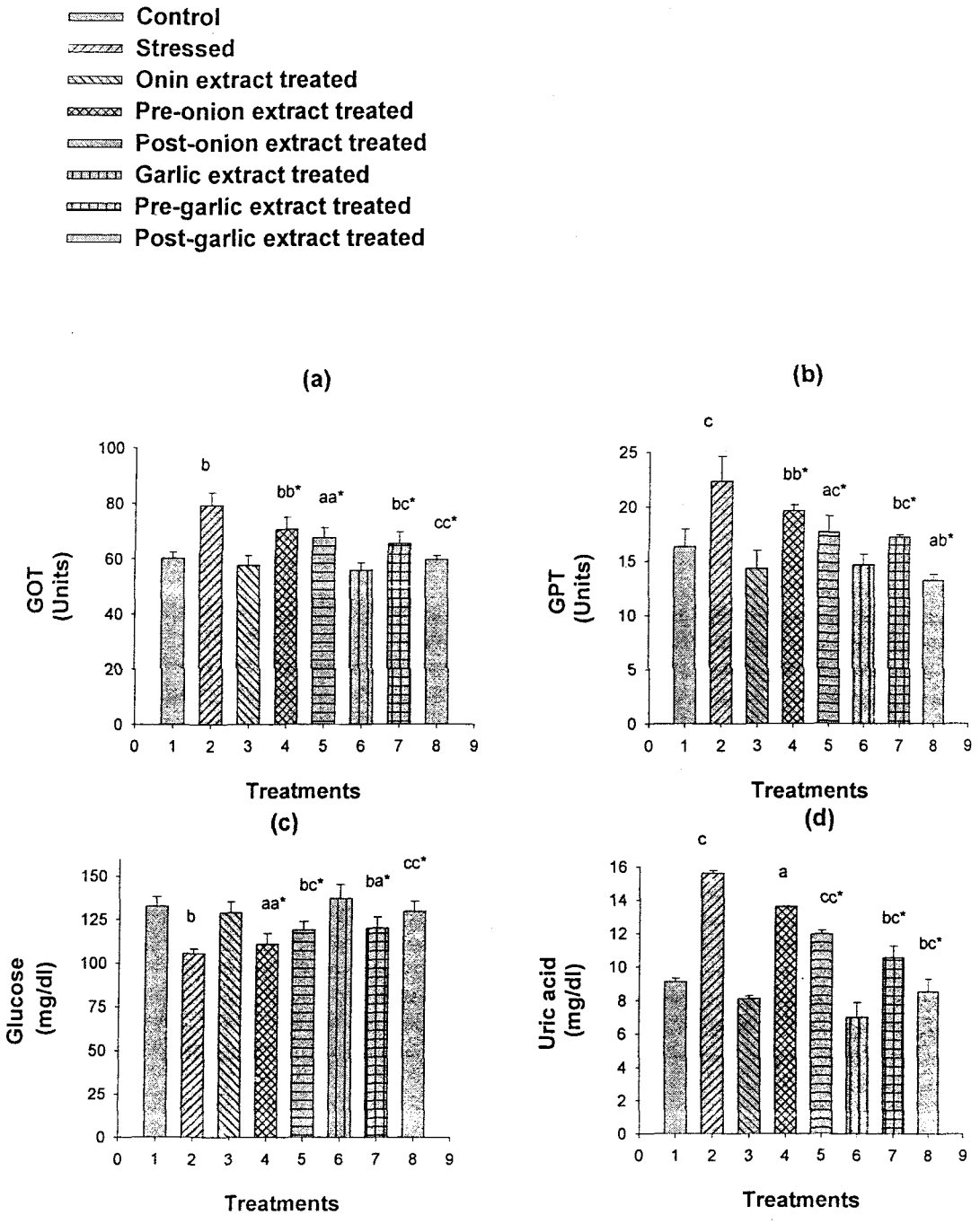
a = p<0.02, b = p<0.05 and c = p<0.001 shows p value as compared to controls where as a* = p<0.02, b* = p<0.05 and c* = p<0.001 shows p values as compared to stressed rats.

Fig- 26. Effect of garlic and onion extracts on immobilization stress induced changes in Heart tissue levels of SOD, catalase, GST and MDA



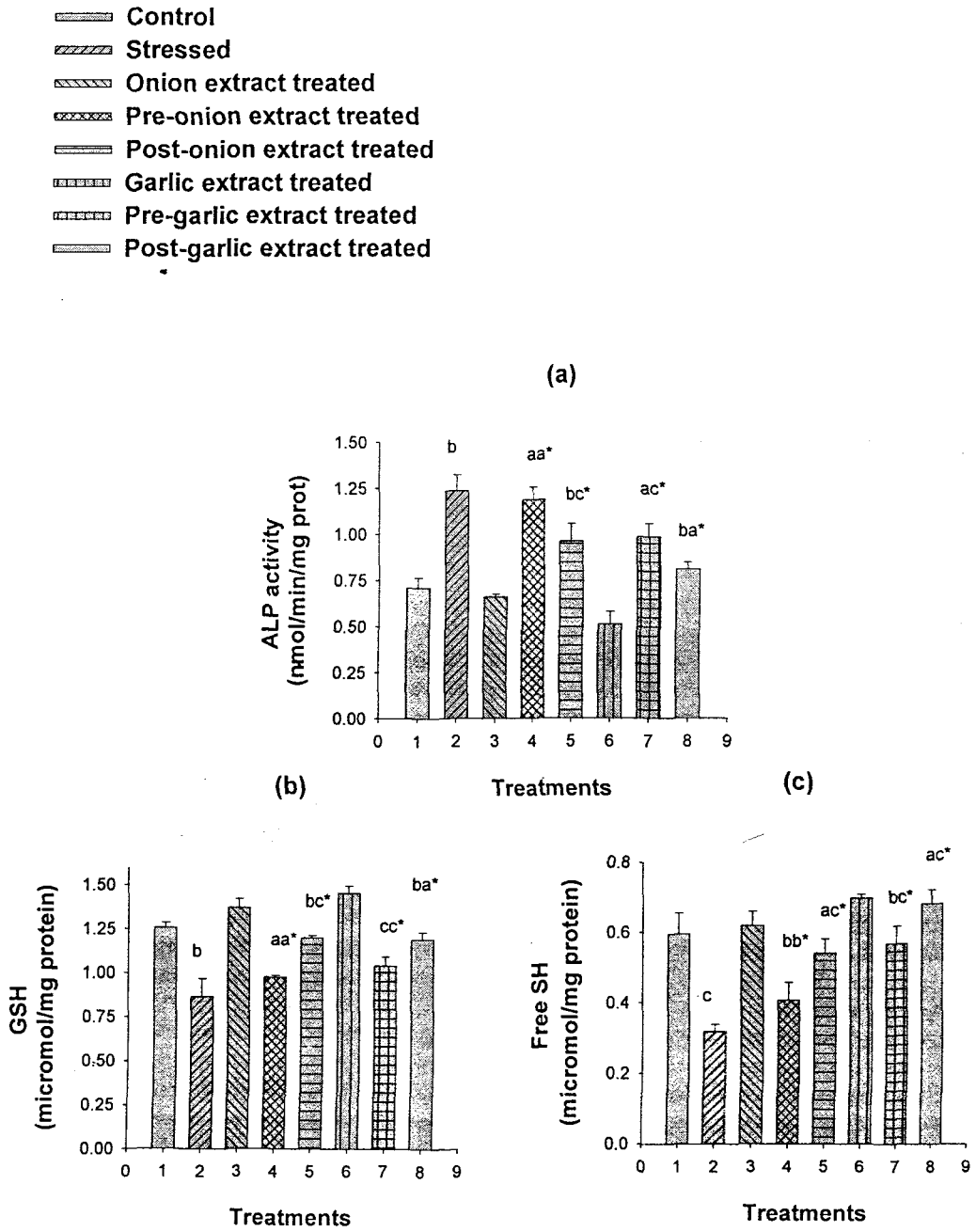
a = p<0.02, b = p<0.05 and c = p<0.001 shows p value as compared to controls where as a* = p<0.02, b* = p<0.05 and c* = p<0.001 shows p values as compared to stressed rats.

Fig- 27. Effect of garlic and onion extracts on immobilization stress induced changes in Heart tissue levels of GOT, GPT, Glucose and uric acid



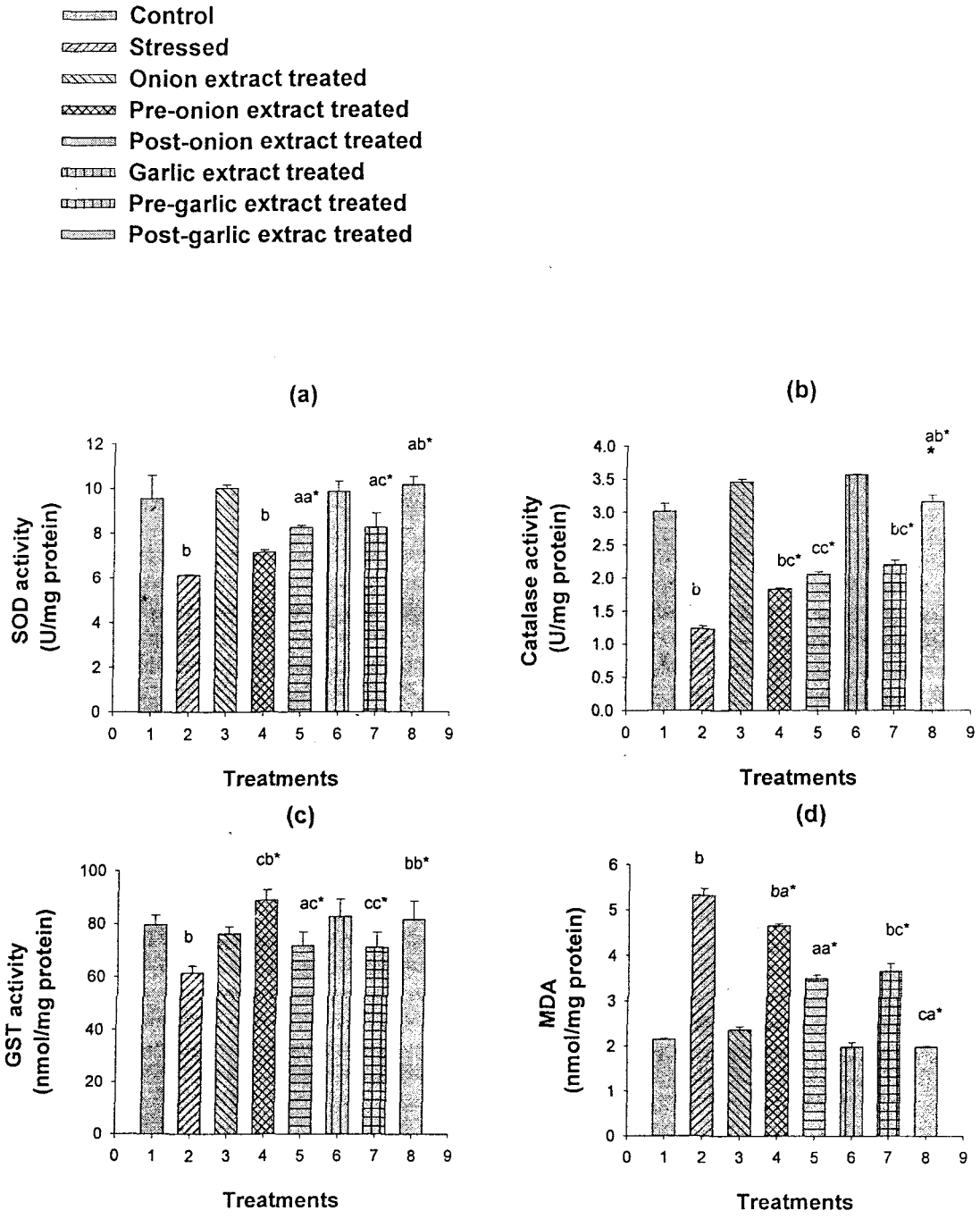
a = p<0.02, b = p<0.05 and c = p<0.001 shows p value as compared to controls where as a* = p<0.02, b* = p<0.05 and c* = p<0.001 shows p values as compared to stressed rats.

Fig- 28. Effect of garlic and onion extracts on immobilization stress induced changes in Heart tissue levels of ALP, Total SH and Free SH groups



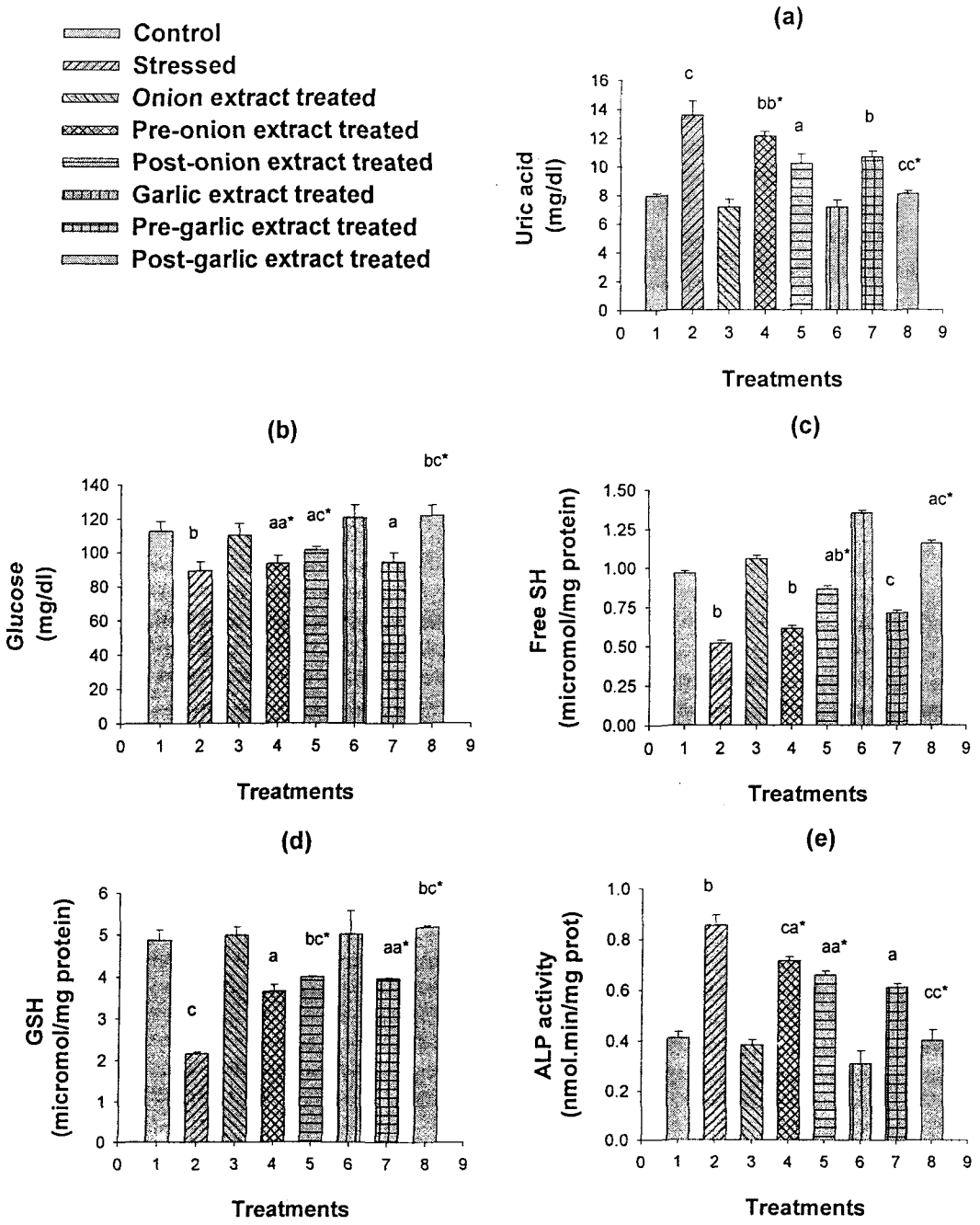
a = p<0.02, b = p<0.05 and c = p<0.001 shows p value as compared to controls where as a* = p<0.02, b* = p<0.05 and c* = p<0.001 shows p values as compared to stressed rats.

Fig- 29. Effect of garlic and onion extracts on immobilization stress induced changes in Kidney tissue levels of SOD, catalase, GST and MDA



a = p<0.02, b = p<0.05 and c = p<0.001 shows p value as compared to controls where as a* = p<0.02, b* = p<0.05 and c* = p<0.001 shows p values as compared to stressed rats.

Fig- 30. Effect of garlic and onion extracts on immobilization stress induced changes in Kidney tissue levels of Glucose, uric acid, ALP, total and free SH groups.



a = p<0.02, b = p<0.05 and c = p<0.001 shows p value as compared to controls where as a* = p<0.02, b* = p<0.05 and c* = p<0.001 shows p values as compared to stressed rats.

DISCUSSION

Controls

In the present study two types of controls have been included, the untreated controls and the vehicle treated controls (normal saline & olive oil). The untreated controls were used for comparing the changes produced by stress alone treatments, while the vehicle (n-saline / olive oil) treated controls were used for comparing the biochemical effects produced by various treatments like vitamins, extracts of *S. nigrum* (Makoi), onion and garlic.

A comparison between vehicle treated controls and untreated controls revealed an insignificant decrease in the activities of free radical scavenging enzymes like superoxide dismutase (SOD), glutathione-S-transferase (GST) and catalase (CAT) with an insignificant decrease in the levels of total and free SH groups, while the level of MDA was found insignificantly increased as compared to untreated controls.

The levels of marker enzymes like ALP, SGOT and SGPT were also found insignificantly increased in the vehicle treated controls as compared to untreated controls.

The insignificant changes in the biochemical parameters observed could be due to handling of animals for oral administration of these vehicles through catheter. The handling of animals is found to induce slight stress (Al-Qirim et al., 2002, Liu et al., 2000).

A single oral dose of olive oil did not show any significant antioxidant effect in the present study. The antioxidant effect of oral administration of olive oil (10%) has been reported in hyperlipemic animals than in normolipemic (Jose et al., 2000). Probably, these antioxidant substances increase their effects in high oxidative stress situations, such as occurs with other antioxidant drugs (Gonzalez et al., 1997).

The normal distribution of free radical scavenging enzyme activities among the tissues revealed highest activities of SOD, GST and catalase in the liver tissues, while the lowest were found in brain. The levels of total and free SH groups were found maximum in liver tissues, while minimum in spleen.

The distribution of marker enzymes ALP, GOT and GPT were found maximum in liver, and heart tissues, while least activities were found in brain tissues. The tissue distributions of the measured biochemical parameters are consistent with earlier findings (Can Verdeth, 1989). Similar to our results previous reports have also shown that brain has poor activity of catalase and moderate activities of glutathione peroxidase and superoxide dismutase (Coyle et al., 1989; Halliwell et al., 1992).

The free radical scavenging enzyme activities and the levels of other biochemical parameters in brain, liver, heart, spleen and kidney tissues were found insignificantly altered by vehicle treatments in comparison to untreated controls, and the changes in tissue level were found similar to their respective alterations in plasma indicating a good correlation between plasma and tissue levels.

It has been observed that the intragastric administration of both the vehicles resulted in slight stress but the effect of stress was observed minimal in olive oil treated rats as compared to n-saline treatments but the changes were insignificant.

Immobilization stress

Immobilization stress is a well-defined method for the production of chronic stress (Kvetnansky et al., 1971). Immobilization stress has also been shown to bring about antioxidant defense changes in the plasma of rats (Liu et al., 1994). Superoxide dismutase, catalase and GST play an important role in scavenging oxyradicals and their products (Mannervik and Danielsin, 1988). In order to maintain the stability of a living organism it is necessary to reach a balance between the oxidative actions and the antioxidant defense. i.e. anti-FRS. Enhanced free radical production with lipid peroxidation has been observed during stress (Clemens, 1991). The decreased activities of SOD, GST and catalase in the present study may be responsible for the elevated free radical levels in stress (Chaudire and Ferrari-Illious, 1999).

The increased MDA levels further confirmed enhanced lipid peroxidation in restraint stress as the MDA levels can be considered as an index of lipid peroxidation (Ohkawa et al., 1979). Malondialdehyde, the three-carbon compound is not exclusively derived from polyunsaturated fatty acids, but also from radical-induced degradation of amino acids, carbohydrates and nucleic acid (Gutteridge and Halliwell, 1990). Thus, immobilization stress generated severe oxidative stress like situation in rats due to combined emotional and physical stress (Sies, 1985).

Effect of vitamin treatments

Many compounds are metabolized by cells causing increased electrophilic radicals that can react with oxygen giving rise to reactive oxygen species (ROS), one of the main sources of free radicals. Paradoxically, ROS can be synthesized in several essential metabolic processes for living cells; however, the alteration in their synthesis can originate oxidation and irreversible cell damage (Baud and Ardaillou, 1986).

ROS are highly reactive and can react with many intracellular molecules, mainly unsaturated fatty acids (phospholipids, glycolipids, glycerides and sterols) and transmembrane proteins with oxidizible amino acids. The oxidation of these molecules causes increased cellular membrane permeability, with consequent alterations of ionic gradients and disruption of several membrane functions and metabolic processes. To counteract the ROS, tissues contain enzymatic and non-enzymatic defense mechanisms, known as antioxidant defense potential (AOP). AOP comprising mainly of SOD, GPx, catalase (enzymatic) and tocopherols, carotenoids, ascorbic acid etc (non-enzymatic) can neutralize free radicals (Greene and Paller, 1991). Thus, lipid peroxidation gets initiated when oxidative stress overcomes the oxidative defenses.

A single oral dose of vitamin A, E and C individually and in combination (vitamin E+C) in low (15 mg/kg body weight) and high doses (50 mg/kg body weight) resulted in an insignificant rise in the activities of

free radical scavenging enzymes and various other biochemical parameters. The treatment of vitamin A, resulted in an insignificant increase in the activities of free radical scavenging enzymes like SOD, GST and catalase, the levels of total and free SH groups were found insignificantly increased, while the level of MDA was found insignificantly decreased by the vitamin A treatments as compared to vehicle treated control, showing decrease in lipid peroxidation and enhancement in antioxidant metabolism.

Recent work has shown that β-carotene, a pigment found in all plants, is most effective quencher of hydroxyl radical and singlet oxygen known in nature and can also function as antioxidant (Burton et al., 1984). β-carotene is known to be major carotenoid precursor of vitamin A. However vitamin A cannot quench singlet oxygen and has a very small capacity to scavenge free radicals (Mathews, 1986), which may account for least antioxidant effect of vitamin A as compared to E and C. While, a great benefit has been observed on childhood mortality in developing countries by vitamin A supplementation (Rahmatullah et al., 1990), due to its role in growth and development.

The protective effect of antioxidant vitamins was reflected by the levels of marker enzymes like ALP, GOT and GPT which were also found insignificantly decreased by the vitamin A, E, C and (E+C) supplementation as compared to vehicle (olive oil) treated controls. The decrease in the activities of marker enzymes too was more by vitamin E and E+C than A and C individually. Thus, the vitamin treatments either individually or in combination caused a decrease in the levels of marker enzymes.

Similar to the results of vitamin A supplementation, vitamin E and vitamin C either individually or in combination (vitamin E+C) resulted in an insignificant increase in the activities of SOD, GST and catalase with increase in the levels of total and free SH groups, while the level of MDA was found insignificantly decreased, when a comparison was made with vehicle (olive oil) treated controls.

Free radicals are produced constantly *in vivo* due to normal cell respiration and the other cellular processes (Spitz et al., 2004). Both *in vivo*

(GSH, SOD, GST, and catalase) and *in vitro* (vitamins A, E, C) antioxidant and free radical scavenging systems exist in the cell to protect it against the damaging effects of free radicals.

Free radicals and free radical reactions are involved in etiology, development and pathogenesis of a number of diseases those which are life limiting (Pryor, 1988). Free radicals like superoxide anion ($O^{\bullet}$-) and hydroxyl radicals ($HO^{\bullet}$) are highly reactive and present a challenge to the cellular, morphological and functional integrity of the cell. It has been reported that diseases result in generation of ($O^{\bullet}$-), but the source of increased superoxide anion is not known, though many intrinsic systems including NADPH oxidase, xanthine oxidase, phospholipase A_2, and mitochondrial enzymes possibly are involved in the generation of superoxide anion (Chen et al., 1997). The reduced levels of MnSOD activity are reported to enhance accumulation of superoxide anion (Yamanaka, 1978; Yamanaka, 1979). Thus, a slightly enhanced activity of SOD by vitamin supplementation can prevent unnecessary accumulation of superoxide anions *in vivo*. Infections, immune response to leukemic leukocytes, initial symptomatic treatment with simple antibiotics /anti-inflammatory drugs may also be the contributory factors for increased superoxide anion (Oberley, 1979).

The consequence of increased free radical generation and imbalances in antioxidant defences is oxidative stress, which leads to oxidative damage, resulting in increased lipid peroxide levels. However, when the tissues respond to oxidative stress by increasing the antioxidant defences, insufficient response may lead to mild oxidative stress, which if prolonged may precipitate several diseases. Antioxidant vitamin supplementation may augment this mild oxidative stress and may have a prophylactic effect.

Among several nutrients, vitamin E plays an important role in scavenging free radicals. Lipophilic vitamin E can act directly with variety of oxy radicals, including peroxy radicals (ROO), CCl_3, $HO^{\bullet}$ (Mac Cay, 1985; Burton et al., 1985), as well as with the superoxide radical ($O^{\bullet}_2$) (Fukuzawa et

al., 1983; Ozawa et al., 1983). Tocopherol can also react directly with singlet oxygen (Fahrenholtz et al., 1974). The effect of vitamin E is found more in enhancing the activities of free radical scavenging enzymes like SOD, GST and catalase with increase in the levels of total and free SH groups than A and C. The tissue sensitivity to the lipid antioxidant effects of vitamin E might have been enhanced by oil as observed in case of E not A (Saccini et al., 1992).

In the present study the level of MDA was found decreased by the vitamin E treatment, this could be due to its antioxidant property as it can transfer its phenolic hydrogen to a peroxyl free radical of a peroxidized polyunsaturated fatty acid (PUFA), thereby breaking the radical chain reaction and preventing the peroxidation of PUFA in cellular and subcellular membrane phospholipids. As a reducing agent, vitamin C reacts with vitamin E radical to yield a vitamin C radical while regenerating vitamin E. Like vitamin E radical, vitamin C radical is not a reactive species because its unpaired electron is energetically stable. A vitamin C radical is converted back to vitamin C by GSH (Fang, 2002). Thus, vitamin C regenerates vitamin E by cooperative interaction (Ramanathan et al., 2005), this may account for maximal antioxidant effect of vitamins (E+C) combined treatment.

Vitamin C (ascorbic acid) is water-soluble and along with vitamin E can quench free radicals as well as singlet oxygen. Ascorbic acid has been shown to react directly with superoxide (Hemila et al., 1985; Nishikimi et al., 1975), hydroxyl radicals (Bielski, 1982), and singlet oxygen (Bodanners, 1979), which results in the increased activities of SOD, GST and catalase. The antioxidant role of physiologic concentrations of vitamin C has been well established. (Aruoma, 1998), vitamin C has protective effect against free radical–induced oxidative DNA damage it has been reported that irradiation increases the oxidation of proteins, amino acids, lipids, vitamin C, and folate in rats. (Hu et al., 1983; Fang et al., 1983). These antioxidants will directly scavenge radicals present in the aqueous compartment. Qualitatively the most important antioxidant of this type is vitamin C (Lewine et al., 1999).

In humans, ascorbate acts as an essential cofactor for several enzymes catalyzing hydroxylation reactions. In most cases, it provides electrons for enzymes that require prosthetic metal ions in a reduced form to achieve full enzymatic activity.

In the presence of transition metals, ascorbic acid can provoke the formation of free radicals. However, there is no evidence that the autoxidative effect of ascorbic acid leads to the promotion of lipid peroxidation (Bendich et al., 1986). In addition to its role as an enzyme cofactor, the other major function of ascorbate is as a key chain breaking antioxidant in the aqueous phase (Jialal et al., 1990). During its antioxidant action, ascorbate undergoes a two-electron reduction, initially to the semidehydroascorbyl radical and subsequently to dehydroascorbate. Two mechanisms have been described by which dehydroascorbate can be reduced back to ascorbate; one is mediated by the selenoenzyme thioredoxin reductase (May et al., 1998) and the other is a non-enzyme mediated reaction that uses reduced glutathione (May et al., 1996). According to the present study the antioxidant effect of vitamin C is found more than vitamin A in terms of measured *in vivo* antioxidant system.

In the present study decrease in the lipid peroxidation was observed by the individual vitamins A, E, C and combined (vitamin E+C) treatments. Significantly increased levels of ethane and pentane in the exhaled air of vitamin E-deficient rats as well as from vitamin C deficient guinea pigs provide further evidence of increased lipid peroxidation when these nutrients are absent from the diet (Kunert et al., 1983; Machlin, 1987). Vitamin E inhibits ROS-induced generation of lipid peroxyl radicals, thereby protecting cells from peroxidation of PUFA in membrane phospholipids, from oxidative damage of plasma very low-density lipoprotein, cellular proteins, DNA, and membrane degeneration. (Topinks et al., 1989). Consequently, a dietary deficiency of vitamin E has been reported to reduce the activities of hepatic catalase, glutathione peroxidases, and glutathione reductase, (Chow et al., 1969) thereby inducing liver lipid peroxidation, and causing neurologic and

cardiovascular disorders, (Muller, 1990; Carr et al., 2000) and all of which were found reversed by dietary vitamin E supplementation. The critical antioxidant role of vitamin E has been supported by increased brain lipid peroxidation and neurodegeneration in mice with a deficiency of α-tocopherol transfer protein (Yokota et al., 2001).

In the present study, the levels of MDA, the end product of lipid peroxidation, were decreased in liver, brain, heart and spleen tissues while the level of GSH were increased by antioxidant vitamin treatments, confirming further their antioxidant role. The decrease in lipid peroxidation may be due to enhanced activities of free radical scavenging enzymes SOD, GST and catalase in these tissues by vitamin treatment. Similar to the results obtained in circulation, the antioxidant effect of vitamin E and E+C combined was predominant in all tissues samples.

Both ascorbate and GSH can react with hydrogen peroxide and oxygen free radicals. It is well known that ascorbate is stabilized by thiols, and this is consistent with the *in vivo* finding of decreased tissue ascorbate levels in GSH deficiency. Deficiency of GSH could conceivably lead to inactivation of other systems that may be involved in reduction of dehydroascorbate or to increased metabolic demand for NADPH. Although the *in vivo* data clearly indicate a function of GSH in the reduction of dehydroascorbate, the enzymology involved needs more study. Possibly dehydroascorbate is oriented *in vivo* at sites favorable for reduction by GSH. Possibly stable GSH-dehydroascorbate complexes are involved. The enhanced GSH levels observed here in response to vitamin C treatment might have facilitated the oxidation of extracellular ascorbate to dehydroascorbate for accumulation in neutrophils (Washko et al., 1993).

Thus, the treatment of vitamin A, E and C individually and in combination (vitamin E+C) resulted in an antioxidant effect, however, the supplementation of combined vitamin (E+C) and vitamin E alone showed maximum antioxidant effect than either vitamin A or C alone. Thus, the antioxidant efficacy of these vitamins in terms of enhancement of free radical

scavenging enzyme activities both in circulation and tissues can be summarized as follows:

Vitamin E+C ≥ vitamin E > vitamin C > vitamin A.

Effect of pre- and post-vitamin treatments individually and in combination on restraint stress induced biochemical changes

All the cells in the body are exposed chronically to oxidants from both endogenous and exogenous sources but are also equipped with an antioxidant system. Reactive oxygen and nitrogen species, if unchecked, can contribute to chronic disease development by oxidatively modifying lipids, nucleic acids and proteins (Liu et. al., 1996). Nutrients, both water-soluble and lipid soluble comprise an important aspect of the antioxidant defense system.

The antioxidant defense potential of vitamin A, E, C individually and in combination (vitamin E+C) was evaluated on modulation of restraint stress (Immobilization stress) induced oxidative stress. The antioxidant potential of immobilization stress treated rats were found reduced both in circulation and tissues, and the MDA levels increased in comparison to controls as discussed earlier. Vitamin (either individual A, E, C or vitamin E+C combination) pre-treatment ameliorated antioxidant defense potential, prevented increase in MDA levels by restraint stress treatment, and increased the antioxidant defense potential by enhancing the activities of free radical scavenging enzymes SOD, GST and CAT. The post-treatment of these vitamins to the restrained rats was found more effective in reverting the stress altered oxidative metabolism to controls. The prevention or reversion of oxidative metabolism was measured in terms of activities of SOD, GST, catalase and levels of GSH and MDA as discussed. The pre or post treatment of vitamin E alone or E+C combined was found most effective in combating restraint stress induced oxidative changes.

Antioxidants can scavenge ROS before they can cause damage to the various biomolecules or prevent oxidative damage from spreading out, e.g. by interrupting the radical chain reaction of lipid peroxidation. The antioxidant

defense systems in the human body are extensive and consist of multiple layers that protect at different sites and against different types of stress. The antioxidant role of vitamin A, E and C has been reported for protection against diseases and degenerative processes. (Yun et al., 2002).

In the present study six hours of restraint stress resulted in decreased activities of free radical scavenging enzymes like SOD, GST and catalase with decrease in the levels of total and free SH groups. Superoxide dismutase protects aerobic organism against the potential deleterious effects of superoxide. The enzyme occurs in several different compartments of the cell. Superoxide dismutase removes $O_2^{\bullet}$ by converting it to H_2O_2, which is mostly removed by catalase and glutathione peroxidase enzymes (Halliwell, 1999).

The removal of free radicals is achieved through enzymatic and non-enzymatic reactions. The principle defense systems against oxygen radicals are SOD, GST, catalase (a heme enzyme), glutathione peroxidase, and glutathione reductase and antioxidant nutrients. Vitamins directly scavenge ROS and upregulate the activities of antioxidant enzymes. Among them, vitamin E has been recognized as one of the most important antioxidants. The levels of marker enzymes like alkaline phosphatase (ALP), glutamate oxalate transaminase (GOT) and glutamate pyruvate transaminase (GPT) was found significantly decreased by both pre- and post-vitamin stress treatments, when a comparison was made with stress alone treatments.

Six hours of immobilization stress resulted in the generation of oxidative stress/ reactive oxygen species (ROS) in the liver, brain, heart, spleen and kidney tissues of the rats by decreasing the levels of GSH and the activities of enzymes SOD, GST and catalase, while enhancing the level of thiobarbituric acid reactive substances (TBARS), which is an indicative of lipid peroxidation. As observed in the circulation the antioxidative effects were observed in the tissues too, which were depicted by the enhanced activities of free radical metabolizing enzymes and decreased levels of MDA by both the pre- and post-treatments of these vitamins. However, the maximum antioxidative effect was found in liver tissues, while minimum but

significant in brain tissue. The post- vitamin stress treatment was found more effective in reducing the stress-induced changes than pre-vitamin stress treatments in terms of alterations of the biochemical parameters measured as mentioned above.

The ROS produced in response to restraint stress may propagate the initial attack on lipid rich membranes of the brain (Das and Khanna, 1997) and other tissues to cause lipid peroxidation (LPO). The enhanced LPO may also be due to marked depletion of GSH content of brain, which acts as one of the guarding factors against oxidative stress (Lewine, 1982). The glutathione depletion might be the result of decreased activities of GST, SOD and catalase, the free radical scavenging enzymes. Restraint stress has been shown to bring about antioxidant defense changes in the plasma of rats (Al-Qirim et al, 2002). The already known poor catalase and moderate glutathione peroxidase and SOD activities of brain (Paya et al., 1992) are found further reduced by restraint stress. GST also possesses peroxidase activity and can directly attack the peroxides that may be generated via oxidative reduction recycling, resulting in decreased toxicity (Prohaska, 1980). But the decreased GST activity as observed in the present study might have further contributed to the enhanced lipid peroxidation as depicted by increased TBARS levels. Thus, immobilization stress is found capable of generating oxidative stress in rat tissues including liver and brain. The generated ROS is considered the major factor involved in the mechanism of liver cell injury (Videla et.al.2003).

Both natural non-enzymatic *exvivo* (vitamins A, E and C) and *invivo* SOD, GST, catalase and glutathione antioxidant defense mechanism exist. However, the antioxidant mechanism fails either due to overproduction of free radicals or decreased activities of scavenging enzymes, or both causing lipid peroxidation. Since lipid peroxidation is a self-propagating chain reaction the initial oxidation of only a few lipid molecules can result in significant tissue damage and disease, especially so in PUFA rich brain tissues. Glutamate, dopamine, iron-catecholamine complexes and reactive

oxygen species are involved in several diseases of the nervous system including Parkinson's disease, Schizophrenia and Alzheimer's disease (Ramanova et al., 1994). In all these diseases oxidative stress and a failure of antioxidant defense are found involved.

The acetylcholinesterase inhibitors are reported to give only a temporary relief in Alzheimer's disease (Geer, et. al., 2003), still definitive clinical trials are required to reduce both neurotoxic, neuroinflammation and oxidative stress. The results of the present study suggest that the dietary supplementation of vitamin E may be effective in combating the neurodegenerative diseases. With initial large doses to build up tissue levels followed by the lessening of supplementation may be effective in both prevention and therapeutic efficacy of these vitamins. Similar to our findings, a high dose of vitamin E dietary supplementation or parenteral vitamin E administration (e.g. vitamin E succinate) is proposed to serve as a successful therapeutic strategy for the prevention or treatment of Parkinson's disease (Farris and Zhang, 2003).

Another important aspect to consider in tissue oxidative damage is the effectiveness of glutathione antioxidant system (Cruzdela et al., 1998). GSH and vitamin C are found to regenerate and spare each other as antioxidants (Martersson et. al., 1991). Moreover, liver vitamin C levels were found to be reduced when GSH levels were decreased by GSH depleting agents or stress (Martersson et. al., 1991). Thus restraint stress not only causes a decrease in the GSH level but also affects vitamin C content of the liver resulting in oxidative stress and membrane damage. Ascorbic acid is a potent scavenger of ROS in plasma and extracellular compartments of the liver (Inoue, 2001). Vitamin C alone is found to be more effective than vitamin A, but less effective than vitamin E in decreasing oxidative stress. This could be due to a highly decreased concentration of glutathione during stress, because of enhanced levels of catecholamines and glucagon too, which are known to reduce glutathione levels (Simmons et. al., 1991), this might also have affected the enzymatic regeneration of ascorbic acid, which occurs at the

expense of reduced glutathione (GSH). While, the combined (vitamin E +C) therapy is found to be most effective in combating oxidative stress generated by restraint stress. The antioxidant effect of vitamin C in scavenging and destroying free radicals in combination with vitamin E is probably mediated through the enhancement of glutathione levels and the activities of SOD, GST and catalase, which is reflected by a decrease in the levels of lipid peroxidation product (MDA) after pre- and post-stress combined vitamins (E+C) treatment. The beneficial effects of the vitamin treatments were clearly confirmed by the reversion of stress altered biochemical marker enzymes GOT and GPT levels towards their control values.

The present study indicates that vitamins E and C when given together have potential antioxidant action both as prophylactic and curative agents on liver, brain and other tissues of rats, and can combat oxidative stress generated by immobilization stress, through modulation of inherent antioxidant metabolism. Increased oxidative stress is found to accompany not only cirrhosis but also various other clinical disorders including AIDS, diabetes mellitus, malnutrition, adult respiratory distress syndrome and Parkinson's disease, and it becomes important to prevent oxidative stress in these patients.

The measurement of disappearance of the endogenous antioxidants in plasma in relation to the formation of lipid peroxides from endogenous lipids has revealed that vitamin C is the only antioxidant that can completely prevent initiation of lipid peroxidation induced by aqueous peroxyl radicals (Frei et al., 1988). Both vitamin C and vitamin E are sacrificial antioxidants that can donate hydrogen atoms (Halliwell and Gutteridge, 1985). Vitamin E, unlike vitamin C is localized in membranes and lipoproteins, where it interrupts the radical chain reaction of lipid peroxidation (Pryor, 1994). Therefore, vitamin E is also called a chain breaking antioxidant.

In addition, studies have suggested that supplementation with at least 100 mg / day of vitamin E may decrease the risk of heart disease (Stampfer and Rimm, 1995). This is well above the current RDA and is far greater than

can be obtained from even a very well balanced diet. These findings have rekindled the debate as to whether dietary recommendations should consider optimal levels of these vitamins rather than the levels needed to prevent lipid peroxidation. As a fat-soluble vitamin, E has the potential for toxicity. However, it does appear to be the least toxic of the fat-soluble vitamins. No instances of toxicity have been reported at doses of less than 1600 mg / day (Das, 1994).

S. nigrum

Solanum nigrum (Makoi) is commonly used in traditional medicine as a remedy for treating various diseases. The plant of *S. nigrum* has been reported to play an adjuvant role in hepatoprotection. Inhibitions of lipid peroxidation and free radical scavenging activities have been suggested as possible mechanism of action (Sarwat et al., 1995). Therefore, the present study was conducted to evaluate the antioxidant effect of aqueous extract of *S. nigrum* leaves on restraint stress induced deranged free radical metabolism.

Any natural or synthetic compound with antioxidant properties may help to alleviate the damage totally or partially. Therefore removing superoxide ion and hydroxyl radical is probably one of the most effective defense mechanisms against a variety of diseases.

A single dose of oral extract (100 mg / kg body weight) of *S. nigrum* (Makoi) leaves resulted in an insignificant increase in the activities of free radical scavenging enzymes like SOD, GST and catalase, while the serum levels of MDA and uric acid were found insignificantly decreased as compared to normal saline treated controls.

An insignificant decrease in the tissue levels of glucose and SH groups were observed by the extract treatments when a comparison was made with controls. The marker enzymes like GOT, GPT and ALP were also found insignificantly decreased by the extract treatment.

The results obtained clearly indicate that if Makoi extract is taken under normal / unstressed conditions, it does not alter either the oxidative

metabolism or the marker enzyme levels significantly. But a slight insignificant increase in the free radical metabolizing enzyme activities may have a beneficial effect if the body is exposed to oxidative stress. This is observed in our further studies when the rats were given an oral dose of Makoi extract prior to immobilization stress exposure, as discussed later.

The oral administration of *S. nigrum* (100 mg / kg body weight) extract both pre-stress and post-stress treatments resulted in a significant increase in the activities of SOD, GST and catalase in circulation and various tissues like brain, liver, heart, spleen and kidney. However, the post-extract stress treatment of *S. nigrum* was found more effective than pre treatment.

As the antioxidant enzymes such as SOD, GST, catalase and GPx are the first line of cellular defense against oxidative injury and decompose O_2 and H_2O_2 before they interact to form more reactive ($OH^{\bullet}$) radicals. The enhanced activity of SOD by makoi extract might have helped in quenching of superoxide ($O^{\bullet}$) and oxygen free radicals, produced by immobilization stress. SOD and catalase enzymes are highly specific in their catalytic mode of actions and they decrease the damaging effects of oxidative stress (Halliwell and Gutteridge, 1985; Mac Millan et al., 1998).

Several reports have indicated that *S. nigrum* herbs are hepatoprotective agents and have shown their efficacy in protecting carbon tetrachloride induced oxidative stress (Karndhikar, 1963; Bardhan et al., 1985), their efficacy may be attributed to their free radical scavenging ability. Thus, the extract of *S. nigrum* possesses strong antioxidant properties under oxidative stress situations as evidenced by the increase in the activities of free radical scavenging enzymes and decrease in the levels of MDA. The antioxidant effect could be due to the presence of flavonoids in *S. nigrum*. The extract is found to contain two flavonoids, apegenin and lutcolin (Hiremath et al., 1996b). A large number of flavonoids including these are known to have strong antioxidant properties (Raj and Shalini, 1999). The metal sequestering property of polyphenolic compounds may be responsible for such an antioxidant effect of makoi extract. The possible mechanism of

cytoprotection is attributed to the hydroxyl scavenging property of *S. nigrum* (Prashant et al., 2001). Thus, *S. nigrum* might have exhibited its antioxidant potential by enhancing the free radical scavenging enzyme activities as observed here and removing hydroxyl radicals, and thus reducing the lipid peroxidation as evidenced by reduced MDA levels. Potential antioxidant therapy should, therefore, include either natural free radical scavenging enzymes or agents, which are capable of augmenting the activity of these enzymes, which include SOD, GST and catalase (Cheeseman and Scater, 1993).

Lipid peroxidation has been implicated in a number of deleterious effects such as increased membrane rigidity, osmotic fragility, decreased cellular deformability, reduced erythrocyte survival and lipid fluidity (Thampi et al., 1991). In our study, restraint stress induced elevated levels of MDA in circulation and various tissues like liver; brain, kidney, spleen and heart of rats were found significantly decreased by pre- and post-treatments of *S. nigrum* extract. The decrease was found more in post extract treatment. These results are in agreement with the observations of previous study (Khan et al., 1997). Superoxide ion and hydroxyl radicals are known to cause marked injuries to the surrounding tissues and organs. Lowered activities of SOD, GST and CAT due to restraint stress exposure may result in the accumulation of these highly reactive free radicals leading to deleterious effects such as loss of cell membrane integrity and membrane function (Reedy et al., 1992; Sheela et al., 1995; Krishnakantha et al., 1993). The decrease in the tissue lipid peroxidation on *S. nigrum* treatment can also be correlated with the elevated SOD and CAT activities, confirming antioxidants effect.

Glutathione (GSH) is a major non-protein thiol in living organisms, which plays a central role in coordinating the antioxidant defense processes in our body, and is involved in the maintenance of normal cell structure and function, probably through its redox and detoxification reactions (Gueeri, 1995). Restraint stress decreased the levels of GSH as compared to untreated controls due to loss of glutathione from the circulation and tissues (Speisky et

al., 1985). The lowered level may be due to increased utilization of GSH by antioxidant enzymes such as glutathione peroxidase, which scavenges H_2O_2 (Anand et al., 1996). The glutathione depleted state, particularly during oxidative stress of hepatocytes causes a rise in the cytosolic Ca^{2+} concentration and in addition leads to protein thiol oxidation. GST and GPx are essential for maintaining a constant ratio of reduced glutathione to oxidized glutathione in the cell. Administering *S. nigrum* leaf extract both before and after the restraint stress treatment helped to restore the GSH levels to near those of controls. The extract maintained the activity of GPx almost at near normal by its ability to increase the level of reduced GSH and decrease lipid peroxidation. Previous studies have also shown increase in the GST activity when rats were fed *S. nigrum* extract (Moundipa et al., 1991).

The antioxidative role of *S. nigrum* leaf extract has also been observed in protection of gastric mucosal injury and inhibition of progression of gastric ulcers (Singh et al., 2002).

Six hours of restraint stress resulted in a significant increase in the circulating and tissue levels of marker enzymes like ALP, GOT and GPT. The activities of GOT and GPT are the most sensitive tests employed in the diagnosis of hepatic diseases (Chenoweth et al., 1962). The increased levels of these enzymes can be attributed to the damaged structural integrity of the hepatic and cardiac cells, because the enzyme ALP is located in the cytoplasm and is released into circulation after cellular damage (Sallie et al., 1991). On administering *Solanum nigrum* leaves extract both before and after restraint stress exposure, decreased levels of serum GOT, GPT and ALP have been observed. This shows that *Solanum nigrum* extract to an extent preserves the structural integrity of the liver and heart from the adverse effects of restraint stress. The efficacy of any hepatoprotective drug is essentially dependent on its ability to reduce the harmful effects / or to maintain the normal hepatic physiology, which has been observed in the present study, depicted by the decrease in the circulating and tissue levels of GOT, GPT and ALP.

The study clearly indicates that the pre-treatment of aqueous leaf extract of *S. nigrum* helps in the maintenance of structural integrity of hepatocytic cell membrane by resisting oxidative stress generation in response to immobilization, while the post aqueous leaf extract treatment may help in regeneration of damaged liver cells by enhancing the free radical scavenging enzyme activities and reduction of MDA levels. Thus, a prophylactic and a predominant curative role of *S. nigrum* has been observed on oxidative stress.

Garlic and Onion

The biological activity of an extract of garlic or onion has been found to depend on its mode of preparation (Shashikant et al., 1986; Kleijnen et al., 1989). Organic solvent extraction or steam distillation can cause inactivation of active ingredients present in garlic and onion (Kleijnen et al., 1989). Thus, in the present study, we prepared an aqueous extract of garlic and onion in a similar manner as it is generally prepared for nutritional dietary use. This choice of preparation method is supported by the observation that the antioxidant and anti-platelet activity of garlic and onion is greater in aqueous extract than in extracts prepared with alcohol or acetone (Mohammad et al., 1986).

Garlic contains various biochemically active substances including thioallyl compounds, and since its extracts have been known to protect organs from various oxidative injuries (Kourounakis et al., 1991; Horie et al., 1992), we investigated the protective effect of aqueous extract of garlic and onion against immobilization stress induced oxidative injury and compared their antioxidant effects. The results of the present study demonstrated that the restraint stress induced oxidative changes in the plasma and tissues were ameliorated by aqueous garlic and onion extract treatments, as evidenced by the changes in the activities of free radical scavenging enzymes, the levels of malondialdehyde and glutathione, and various biochemical parameters like glucose, uric acid, and marker enzymes (ALP, GOT and GPT). These

findings suggest that garlic and onion extracts have a protective role in oxidative injury, which may be attributed to its antioxidant effects.

An insignificant increase in the circulating levels of SOD, GST, catalase, total and free SH groups and glucose, with an insignificant decrease in the concentration of MDA and uric acid was found in the garlic and onion extract (100 mg/kg body weight) treated rats as compared to n-saline treated controls. Both garlic and onion extracts did not show significant antioxidant potential in the absence of oxidative stress, similar to Makoi extract treatment alone, but their antioxidant effect was exhibited in the presence of immobilization stress.

The antioxidant enzymes SOD, GPX and catalase play an important role in maintaining physiological levels of oxygen and hydrogen peroxide. The SODs are a group of enzymes that rapidly catalyze the conversion of O_2 to H_2O_2 and oxygen. GPx is a major enzyme that inactivates a variety of organic peroxides and thus controls the cellular peroxide levels. It also reduces H_2O_2 to oxygen and water (Park et al., 1994). Several studies have indicated that treatment of endothelial cells with SOD / CAT decreases damage caused by free radicals. (Becker et al., 1994; Crawford et al., 1991; Leff et al., 1991; and Freeman et al., 1982). The results of the present study showed that slightly increased activities of SOD, catalase and GST by the garlic extract treatment might hasten the dismutation of O_2 and decomposition of H_2O_2.

The levels of marker enzymes like ALP, GOT and GPT were found insignificantly decreased by the garlic and onion extract treatments as compared to vehicle treated controls.

The antioxidant property of onion lies in its organosulfur / lipid soluble antioxidant compounds like diallyl sulfides, oxides, and thiols which can trap electrons. Thus it scavenges many free radicals including hydroxyl radicals (Klanns-Dieter, 1983).

In the present study the antioxidant effects of garlic and onion in absence of stress were also evaluated on liver, heart, brain, spleen and kidney tissues. Similar antioxidant pattern was seen in tissues as observed in

circulation. There was an insignificant increase in the free radical scavenging enzymes with increase in the levels of glucose, while the levels of uric acid and MDA were found insignificantly decreased. However, the antioxidant effectiveness of both the extract from high to low on various tissue samples can be summarized as follows:

Liver > Kidney > Heart > Brain > Spleen

Effect of aqueous extract of garlic and onion on restraint stress induced changes in rat plasma and tissues

A single oral dose (100 mg / kg body weight) of aqueous extract of garlic and onion was given to rats, both before (pre-extract stress treatment) and after (post-extract stress treatment) 6 h of stress exposure. The levels of free radical scavenging enzymes like SOD, GST and catalase were found significantly decreased by the stress exposure which reverted to their normal control values by the garlic / onion treatment both prior to and after stress. This could be due to the organosulfur compounds present in the garlic and onion, as aqueous garlic extract is reported to contain allicin, alliin (Weinberg et al., 1993) and two major organosulfur compounds SAC, SAMC which are found to have free radical scavenging activities (Naito et al., 1981). These antioxidant compounds might have exerted their effects probably through enhancing the activities of free radical scavenging enzymes as observed in the present study and by modulating the glutathione levels and GST activity as observed by others (Horie et al., 1992). Similar to our observation increased glutathione content and SOD; GST activities are reported in cardiac muscles after garlic ingestion (Banerjee et al., 2002).

The levels of MDA, an end product of lipid peroxidation, were significantly increased in plasma and tissues after 6 h of restraint stress. This observation is in agreement with previous studies, in which elevated levels of lipid products were decreased from 40 to 80% above basal values (Daryani et al., 1990; Cetinkale et al., 1999). Restraint stress resulted in an enhancement of lipid peroxidation in rats, which is directly related to free radical mediated

toxicity. Oxidative stress produced may then be amplified and propagated by an auto catalytic cycle of metabolic stress, tissue damage and cell death, leading to further increase in free radical ($O_2^{-\bullet}$, $OH^{\bullet}$, H_2O_2) production and depletion of antioxidants (Baynes, 1991). The increased concentration of lipid peroxidation products observed in restraint stress is also associated with decreased activities of free radical scavenging enzymes such as SOD, GST and catalase. A decrease in the activities of these enzymes can lead to the excessive availability of superoxides and peroxy radicals, which in turn generate hydroxyl radicals resulting in the initiation and propagation of lipid peroxidation (Sacks et al., 1978). The targets of oxidative damage are usually critical biomolecules such as nucleic acids, proteins, and lipids (Gutteridge et al., 1990). The present study demonstrated that aqueous extracts of garlic and onion inhibited significantly the elevation of MDA levels both in circulation and in tissues like liver, brain, heart, spleen and kidney in response to stress and reverted it back to near control values. Thus, garlic extract could be protective against distant organ damage by preserving the cellular integrity. Aqueous extract of garlic appears to be involved in both the early (pre-treatment) and late (post-treatment) phases of oxidative damage to improve the tissue response.

Flavonoids and phenolic compounds are the most predominant components of onion and garlic (Dorant et al., 1996). These compounds have free radical scavenging and antioxidant activities. Onion extracts have shown potential antimutagenic activity (Kendler et al., 1987). The onion extracts had phenolic hydroxyl groups and antioxidants of phenolic groups have been recognized to function as electron or hydrogen donors (Shahidi and Wanasundara, 1992). The role of antioxidants has attracted much interest with respect to their protective effect against free radical damage that may be the cause of many diseases including cancer (Nakama et al., 1993). The antioxidative effect of onion extract is mainly due to the phenolic components, such as the flavonoids (Pietta et al., 1998). Some flavonoid and non-flavonoid compounds have been reported to also show alkylperoxyl

radical scavenging activity thus reducing radical-mediated pathogenesis, e.g. carcinogenesis (Sawa et al., 1999). It may be possible to limit oxidative stress-induced tissue damage and, hence, prevent or ameliorate disease progression by favoring the balance towards lower oxidative stress.

Glutathione is an important constituent of intracellular protective mechanisms against various noxious stimuli including oxidative stress. However, reduced glutathione as the main component of endogenous non-protein sulfhydryl pool is known to be a major low molecular weight scavenger of free radicals in the cytoplasm (Ross, 1988; Shaw et al., 1990). Because of their exposed sulfhydryl groups, non-protein sulphydryls bind a variety of electrophilic radicals and metabolites that may be damaging to cells (Szabo et al., 1992). The results of the present study further support the notion that depletion of tissue GSH is one of the major factors that permit lipid peroxidation and subsequent tissue damage.

The extract of onion also showed similar but less effective (than garlic) antioxidant property. This could be due to differences in the constituents of garlic and onion. Garlic has allicin, alliin, SAC and SAMC, which act as strong antioxidants, while onion has organosulfur compounds having weak antioxidant property (Horie et al., 1992).

The activity of GOT and GPT are sensitive indicators of acute hepatic necrosis, and the ALP level is known to be indicative of hepatobiliary disease (Kaplan et al., 1987). In the present study, the restraint stress caused a significant increase in circulating GOT, GPT and ALP levels. The increase of these enzymes indicates degenerative changes and hypo function of heart and liver (Kaplan et al., 1987). The liver and heart are considered to be the principle target organs for restraint stress. Thus, the functions of both heart and liver are found adversely effected by restraint stress as indicated by altered levels of their marker enzymes. Moreover, the increased level of GOT, GPT and ALP in restraint stress clearly showed a harmful and stressful influence on the hepatic, and cardiac tissues which is consistent with those reported in aflatoxicosis and several other diseases (Miller et al., 1994). The

restraint stress induced increase in the activities of these marker enzymes were reverted to their normal control values by both pre and post garlic and onion extract treatments. The post-treatment of both the garlic and onion extracts was found more effective than pre-treatment. However, the garlic extract showed more antioxidant effect than onion both in circulation and tissues.

Plasma and tissue levels of glucose were found significantly decreased in response to restraint stress. This could be due to the enhanced catecholamine levels due to restraint stress (by stress induced adreno-medullary firing). The catecholamine secretion evokes an initial repression of insulin secretion, followed by later rebound hypersecretion of the pancreatic hormone and a consequent hypoglycemia (Said et al., 1987), such a situation may be responsible for decreased plasma glucose levels seen during restraint stress, either by enhancing peripheral glucose uptake or by interacting directly with β-cells of pancreas (Karunayaka et al., 1984). Six hours of immobilization stress resulted in decreased glucose concentration in plasma and tissues with decreased free radical scavenging enzyme activities, which might have further contributed in aggravating the oxidative stress, because glucose is also a scavenger of OH radicals, having a rate constant comparable with mannitol (Halliwell and Gutteridge, 1990). Other studies have also shown that restraint stress significantly decreases circulating glucose levels. (Quirce et al., 1981). The stress induced decreased concentration of glucose were reverted to their control values by both garlic and onion pre- and post-treatments, which could be due to decreased stress metabolism.

Uric acid is considered as non-enzymatic antioxidant, but increased production of uric acid means increase in free radical production due to activation of the xanthine oxidase enzyme system (Nemeth et al., 2002). In the present study uric acid concentration has been observed significantly raised in restraint stress exposure. The increase in the uric acid concentration in response to restraint stress could be detrimental, as GSH contents were also found decreased. The treatment with garlic and onion extracts resulted in a

significant decrease in the uric acid level in both pre and post extract treatments with a relative dominance by later. The role of uric acid as an antioxidant or pro-oxidant is controversial. In some diseases the increased concentration of breakdown products of uric acid have been observed, suggesting that uric acid does indeed react with some oxidants *in vivo* (Grootveld et al., 1987). However, reaction of uric acid with certain oxidizing species such as hydroxyl radical ($HO^{\bullet}$) or peroxyl radicals can generate uric acid radicals that are themselves capable of doing biological damage (Arouma et al., 1989). The increase in uric acid concentration due to restraint stress could be due to body's natural response to combat enhanced free radicals produced due to decreased activities of its scavenging enzymes or because of enhanced xanthine oxidase activity observed during oxidative stress (Nemeth et al., 2002) / or also because of high levels of catecholamines, as some studies have shown that catecholamines increase during purine catabolites (Yonetani et al., 1979).

A strong prophylactic antioxidant effect of garlic extract is observed than onion. As prevention is better than cure, garlic extract can be used as a nutritional supplement to patients for preventing further development of oxidative stress / or can be taken as a preventive measure as no side effects of garlic have been reported.

SUMMARY & CONCLUSION

Two types of controls were included in the present study, n-saline or olive oil (vehicle) treated controls used for comparing the effects of *S. nigrum*, garlic, onion extracts and vitamin A, E and C treatments respectively; untreated controls were used for comparing the effects of immobilization stress on various biochemical parameters. The n-saline or olive oil treated controls showed a slight decrease in the circulating activities of free radical scavenging enzymes SOD, GST, catalase and the levels of reduced glutathione, with increase in the levels of marker enzymes like ALP, GOT and GPT. While the levels of MDA were found insignificantly enhanced in comparison to untreated controls. The changes observed may be due to mild stress generation as a result of handling of rats for vehicle treatments. A single oral dose of olive oil did not show any significant antioxidant effect.

Restraint or immobilization stress is a simple model of inducing both emotional and physical stress (Kvetnansky and Mikulaj, 1970). With the increase in the duration of stress exposure re from 2 h to 24 h, a significant gradual decrease in the circulating activities of SOD, GST and catalase was observed, while the levels of MDA were found significantly enhanced. Thus, immobilization of rats induced a significant oxidative stress causing a decrease in free radical scavenging enzymes and increase in lipid peroxidation, as indicated by increased MDA levels.

A potential role for the antioxidant micronutrients vitamins A, E and C in modulating oxidative stress generated by restraint stress may determine their clinical usefulness as supplemental nutritional therapeutic agent in disorders affecting free radical metabolism. The intragastric administration of antioxidant vitamins A, E and C either individually or in combination (vitamin E+C) resulted in an insignificant increase in the circulating and tissue levels of SOD, GST, catalase and total and free SH groups, while the levels of MDA and marker enzymes alkaline phosphatase, GOT and GPT activities were found insignificantly decreased as compared to vehicle treated controls. The results indicated that in the absence of oxidative stress these antioxidant vitamins do not alter the *invivo* antioxidant system of cells significantly, but caused only an

insignificant enhancement of free radical scavenging enzyme activities and decrease of lipid peroxidation. Thus, they can be taken as a preventive measure to take care of the normal oxidative stress faced by cells due to oxidative metabolism.

The activities of free radical metabolizing enzymes SOD, GST and catalase were observed insignificantly enhanced by the treatment of aqueous extracts of *S. nigrum*, garlic and onion. The levels of SH groups and glucose were found insignificantly increased, while the levels of uric acid and MDA were insignificantly decreased as compared to n-saline treated controls. The levels of marker enzymes were also found decreased by the treatment of all these three extracts. Similar to antioxidant vitamin treatments, these extracts too did not show significant antioxidant effect in the absence of oxidative stress. No toxic effect is observed by the intragastric administration of these extracts, as depicted by the insignificantly decreased levels of marker enzymes.

The modulation of 6 h of immobilization induced oxidative stress was studied by both pre- and post-intragastric administration of the antioxidant vitamins and the aqueous extract of *S. nigrum*, garlic and onion.

A significant increase in the circulating and tissue activities of SOD, GST and catalase and the levels of SH groups was observed by the vitamins A, E, C and treatments both before and after 6 h of stress exposure, showing a decrease of oxidative stress (Zaidi et al., 2003). The significant decreased levels of MDA by vitamin treatment confirmed a decrease in lipid peroxidation. The circulating and tissue levels of ALP, GOT and GPT, which were enhanced due to stress, were also found decreased by the vitamin treatments. Though both pre- and post- treatment of these vitamins were found effective in restoring the stress altered free radical scavenging system towards their control values, a predominant effect of post-vitamin treatment was observed. The efficacy of antioxidant potentials of these vitamins in terms of measurement of above mentioned biochemical parameters can be summarized as follows:

Vitamin (E+C)> vitamin E> vitamin C> vitamin A

The treatments of rats prior to stress exposure by aqueous extract of *S. nigrum* leaves showed a resistance to stress induced decrease of SOD, GST and catalase activities. The circulating and tissue levels of SH groups and glucose were significantly enhanced, while the levels of MDA and uric acid were significantly decreased. The post-extract treatment resulted in a significant reversion of the stress altered biochemical parameters towards their respective control values. The post-treatment was found more effective than pre-treatment in combating the oxidative stress depicting a predominant curative role of *S. nigrum* extract.

Aqueous extracts of onion and garlic showed a protective action against restraint stress induced oxidative stress in the rats as evidenced by lowered lipid peroxidation (decreased MDA levels) and elevated levels of *invivo* enzymatic (SOD, GST and catalase) and non-enzymatic (GSH and glucose) antioxidants. In addition decreased levels of circulating and tissue ALP, GOT and GPT demonstrated that the prior treatment of both the extracts prevented the stress induced tissue damage. The post-treatment of these two extracts reverted the stress induced altered biochemical parameters towards their respective control values. From the results it could be concluded that the post-garlic extract treatment was found most effective than its own pre-treatment or the pre and post-onion extract treatments.

The antioxidant effect exhibited by both garlic and onion can be attributed to the presence of various antioxidant organosulfur compounds in them, which act as precursor of GSH. The antioxidant effect of these extracts is executed by enhancing the activities of free radical scavenging enzymes, and thus decreasing the lipid peroxidation. Garlic extract showed a pronounced antioxidant effect probably due to the presence of various flavonoids, allicin, alliin, SAC, SAMC while onion has organosulfur compounds. The efficacy of these extract treatments from maximum to minimum can be summarized as follows:

Post-garlic > Pre-garlic > Post-onion > Pre-onion

Thus, oxidative stress generated either as a consequence or an effect of various diseases, ageing, physical or emotional stress can be alleviated by the therapeutic ingestion of antioxidant vitamins, A, E, C or E+C together (Showed maximum antioxidant effects) or aqueous extracts of *S. nigrum*, garlic and onion. Both garlic and onion are routinely used in Indian cuisine. None of these antioxidants showed any side effects as evidenced by the levels of marker enzymes.

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Modulation of restraint stress induced oxidative changes in rats by antioxidant vitamins

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Abstract

In the present study we examined immobilization stress-induced antioxidant defense changes in rat plasma and also observed the antioxidant effects of pre and post vitamins A, E and C administration (15 mg/Kg of body weight) individually and in combination (vit E + C) on these alterations.

Following immobilization stress the circulating activities of superoxide dismutase, catalase and glutathione-S-transferase were decreased, while the level of thiobarbituric acid reactive substances (TBARS) was increased as compared to non-stressed control rats.

Post treatment with individual vitamins A, E and C (after exposure to stress) resulted in a less marked alteration of plasma TBARS levels and activities of SOD, GST and catalase as compared to pre vitamin stress or stress alone treatments. Both pre and post vitamin treatments were effective in preventing stress induced derangement of free radical metabolism with a relative dominance by latter. The combined treatment with vitamin E and C did not show any additive antioxidant effect on restraint stress induced altered free radical metabolism, rather a predominant effect similar to vitamin E alone was observed. The prevention of oxidative stress generated in response to restraint stress by the vitamins can be summarized as: vitamin (E + C) i.e. vit E > vit C > vit A, thus combined vitamin (E + C) treatment though showed maximum preventive effect, but was similar to vitamin E treatment alone, in terms of the circulating activities of SOD, GST, catalase and TBARS levels.

Keywords: Restraint stress; Vit A; Vit E; Vit C; Superoxide dismutase; GST; Catalase

1. Introduction

Immobilization/restraint stress is an easy and convenient method to induce both psychological (escape reaction) and physical stress (muscle work) resulting in restricted mobility and aggression [1,2]. Recently various stresses have been associated with enhanced free radical generation causing oxidative stress [3]. One of the most important consequences of the generation of free radical is the peroxidation of membrane lipids.Moreover, stress has been suggested to decrease the level of glutathione (GSH) and vitamin C which play an important role in protection of tissues from oxidative damage [5,6]. Next to the huge group of polyphenols,especially tocopherols, ascorbic acid and carotenoids have been associated with antioxidative properties [7].

In the present study the oxidative stress generated by the restraint stress was measured in terms of free radical scavenging enzyme activities like superoxide dismutase, catalase, glutathione-S-transferase and thiobarbituric acid reactive substances (TBARS).The antioxidative potential of vitamin A, E, C alone and in combination (vit E + C) was also studied on restraint stress induced oxidant/pro-oxidant status of rats.

The results of the study are likely to contribute to understanding the potential of antioxidant vitamins in preventing/alleviating stress induced diseases involving oxidative damage to cellular constituents.

2. Methods and materials

For the present study male Wistar rats weighing 180–200g were selected, the animals were housed in groups cages,purina diets and tap water were supplied to them ad libitum. Prior to the commencement and throughout the experiment the rats were housed at 24±3°C room temperature and 12 h light/dark cycles. All the reagents and chemicals were purchased from commercial sources.

Immobilization stress was accomplished by placing individual animals in wire mesh cages of their size attached to a wooden board. The rats were deprived of food and water during stress exposure [8]. The animals were subjected to

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Table 1
Effect of Vitamin A, E and C treatments individually and in combination (vitamin E + vitamin C) on the circulating levels of SOD, GST, catalase and lipid peroxidation in restraint stress treated rats (values are ± SEM)

		SOD unit mg of protein	Catalase U/mg of protein	GST U/mg of protein	MDA nmoles/ litre
Control rats (10)		5.44 ± 0.351	0.60 ± 0.114	0.50 ± 0.170	0.64 ± 0.035
Stress treated rats alone (10)		3.22^{d} ± 0.291	0.40^{d} ± 0.02	0.23^{d} ± 0.017	0.97^{d} ± 0.043
Pre vitamin stress treated rats (40) 10 rats each	Vit A	3.45^{a} ± 0.21	0.47^{c} ± 0.022	0.30^{b} ± 0.034	0.76^{a} ± 0.024
	Vit E	3.8^{b} ± 0.070	0.52^{a} ± 0.016	0.42^{a} ± 0.037	0.77^{b} ± 0.030
	Vit C	3.55^{a} ± 0.153	0.44^{b} ± 0.020	0.29^{c} ± 0.024	0.72 ± 0.031
	Vit (E + C)	3.86^{b} ± 0.129	0.53^{a} ± 0.011	0.49^{a} ± 0.024	0.68^{a} ± 0.022
Post vitamin stress treated Rats (40) 10 rats each	Vit A	3.50^{c} ± 0.116	0.55^{d} ± 0.011	0.36^{c} ± 0.030	0.68 ± 0.035
	Vit E	4.2^{d} ± 0.24	0.53^{c} ± 0.013	0.43^{b} ± 0.036	0.65 ± 0.02
	Vit C	4.01^{c} ± 0.20	0.49^{c} ± 0.015	0.32^{d} ± 0.025	0.53^{d} ± 0.028
	Vit. (E + C)	4.42^{d} ± 0.125	0.53^{c} ± 0.016	0.40^{c} ± 0.033	0.63^{b} ± 0.022s

No. of experimental rats are indicated in the parenthesis. ^{a} a' $p < 0.05$, ^{b} b' $p < 0.02$, ^{c} c' $p < 0.01$, ^{d} d' $p < 0.001$ where a, b, c, d as compared to non stressed control rats where a', b', c', d' stressed treated rats.

6hr stress then sacrificed after 30 min by giving sodium pentobarbital (i.p., 50 mg/Kg of body weight). Controls were handled at the same time as the stressed animals and were placed in individual cages during the corresponding time.

For the stress and vitamins A, E, C and E + C treatment studies the animals were divided into two broad groups,one received vitamins (15mg/Kg of body weight) dissolved in olive oil 30 min prior to 6 hr stress session (pre vitamin stress treated), while the other groups received individual vitamins (A, E, C) and combined vitamins (E + C) 30 min after the stress session (post vitamin stress treated).

The rats were sacrificed 30 min after the termination of experiment by giving Sodium Pentobarbital (15mg/Kg of body weight, i.p) and immediately exsanguinated. The heparinized blood was centrifuged (5000 rpm, 15 min) and plasma was quick frozen and stored at −40°C until assay.

The plasma was subjected for the assay of Catalase [9], SOD [10], Glutathione-S-Transferase [11] and thiobarbituric acid reactive substances [12] by standerdised methods. The protein contents were estimated by the method of Lowry et al [13].

3. Statistical analysis

Rigorous statistical analysis were performed for the control/baseline levels of the enzymes under study with respect to the treatments given to the rats. It was performed using one way ANOVA test at $p = 0.05$ on the data obtained by the repeated investigations. Paired t tests were also performed at $p = 0.05$ to decide either the results are significantly different or not, followed by pair wise comparison (Tukey's honestly significant Post hoc analysis). Similar statistical treatments were also given to the data obtained for the enzyme activities from pre and post vitamin stress treatments with respect to the stress alone or non-stresed controls. The results obtained are summarized in Table 1.

4. Results

Six hours of immobilization stress resulted in a significantly decreased circulating activities of SOD ($F_{1,9} = 6.658$ $p = .018$), GST ($F_{1,9} = 148.04$ $p = .02$) and catalse ($F_{1,9} = 61.99$ $p = .001$) while the level of malondialdehyde were significantly enhanced ($F_{1,9} = 34.63$ $p = .0001$) in comparision to non-stressed control rats.The individual vitamin A,E, and C and combined (vit E + C) treatment both prior to (pre vitamin stress treated) or after stress (post vitamin stress treated) resulted in a less significant alteration of these parameters if compared with stressed or non-stressed controls. i.e a reversion towards control values was observed. The post vitamin treatments were found more effective than pre vitamin treatments in reverting the stress induced altered SOD, GST, catalase and TBARS levels towards their control values. However, the post stress oral administration of vitamin E (15mg/kg of body weight) was found more effective in restricting the stress induced decrease of SOD ($F_{1,9} = 8.57$ $p = .002$), GST ($F_{1,9} = 43.37$ $p = .03$), catalase ($F_{1,9} = 28.37$ $p = .005$) and increase of TBARS ($F_{1,9} = 41.14$ $p = .002$) as compared to stress alone or other vitamin treatments. While the combined vitamins (E + C) treatments did not show any additive effect rather an effect similar to vitamin E alone was observed.

5. Discussion

In the present study an attempt has been made to evaluate the effect of antioxidant vitamins A,E and C alone and in combination (vit E + C) on modulation of restraint stress induced oxidative changes in terms of the measurements of circulating activities of SOD, GST, catalase and TBARS levels in rats.

Immobilization/restraint stress is a well known method for the production of chronic stress [14]. This study was of interest as human beings are exposed to both emotional and

physical stress daily in their life [15]. The circulating activities of SOD, GST and catalase were decreased while the level of TBARS was increased in stress treated rats as compared to unstressed control group. Various antioxidants and free radical scavenging enzyme systems exist in the cell to protect it against the damaging effects of free radicals produced as a part of normal cell respiration and other cellular processes [16]. Free radicals and free radical reactions are involved in the etiology and development of a number of diseases, especially those that are life limiting [17]. Restraint stress has been shown to bring about antioxidant defense changes in the plasma of rats [18]. Superoxide dismutase, catalase and GSH play an important role in the detoxification of oxyradicals and their products [19]. In order to maintain the stability of a living organism it is necessary to reach a balance between the oxidative actions and the antioxidant defense i.e anti-FRS. Enhanced free radical production with lipid peroxidation has been observed during stress [20]. The decreased activities of SOD, GST and catalase as observed in the present study may be responsible for the elevated lipid peroxidation as represented by increased TBARS levels is stress [21]. Thus, restraint stress is found capable of generating severe oxidative stress in rats.

Both enzymatic in vivo SOD, GST, catalase [9,10,11] and non enzymatic (vitamin A, E and C) natural antioxidant defense mechanisms exist. However, the antioxidant mechanism fails either due to excessive production of free radicals or decreased activities of scavenging enzymes, or both causing lipid peroxidation. Since lipid peroxidation is a self propagating chain reaction the initial oxidation of only a few lipid molecules can result in significant tissue damage and diseases. A potential role for the antioxidant micronutrient (vitamin C, vitamin E and vitamin A) in modulating oxidative stress thus generated may determine their clinical usefulness. The treatment of rats both prior or after stress with these vitamins showed an increase in the activities of SOD, GST and catalase with a decrease in lipid peroxidation. Post vitamin treatment was found more effective than pre treatment in alteration of these parameters towards their control values. Vitamin A, E and C act as an effective antioxidant of major importance for protection against diseases and degenerative processes caused by oxidative stress [22]. In the present study these vitamins showed their antioxidant potential as radical scavengers by enhancing the activities of SOD, GST and catalase and thus decreased lipid peroxidation as reported by others too [23].

The antioxidant potential of vitamins (E + C) together though was found effective but not additive as expected, rather a predominant effect of vitamin E alone was observed.Vitamin E appears to be the first line of defense against peroxidation of polyunsaturated fatty acids present in cellular and subcellular membrane phospholipids. Vitamin C and E have long been associated with stress. A redistribution of vitamin C has been reported during stress with localized enhanced concentration near adrenal S. Increased knowledge in the use of micronutrients like Vitamin A, E and C as an antioxidant under normal and stressful conditions will have an impact on both clinical and dietetics practice and public health nutrition guidelines.

6. Conclusion

Restraint stress was found to induce oxidative stress through decrease of SOD, GST, catalase and increase of lipid peroxidation as shown by enhanced thiobarbituric reactive substances levels. The pre and post-stress oral administration of antioxidant vitamins A,E and C individually and in combination (vit E + C)were effective in protecting restraint stress induced oxidative changes. The combined vitamin treatment did not show any additive effect, but the post stress vitamin treatments were found more effective than pre vitamin treatments, as far as the alteration in the activities of free radical metabolising enzymes and lipid peroxidation was concerned. Vitamin E treatment was found most effective in preventing/restoring the stress induced decrease of SOD, GST, catalase activities and increase of MDA levels.

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Antioxidant potential of vitamins A, E and C in modulating oxidative stress in rat brain

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Abstract

Background: Stress is known to affect synaptic plasticity, dendritic morphology and induces neurotoxic damage in humans, probably through generation of free radicals. Both ex vivo antioxidant vitamins and in vivo free radical scavenging enzymes exist. In the present study, restraint stress induced pro-oxidant status of rat brain was evaluated in terms of measurement of glutathione (GSH), lipid peroxidation (thiobarbituric acid reactive substances, TBARS) and free radical scavenging enzymes activities. The efficacy of antioxidant vitamins A, E and C alone and in combination was also evaluated in modulating inherent antioxidant system in stressed rats. *Methods*: Rats were treated with vit A, E and C alone (15 mg/kg of body weight) and in combination vitamins (E and C) prior to and after 6 h of restraint stress exposure. Both nonstressed and stressed rats were handled simultaneously. Pro-oxidant status of brain tissue was evaluated by determining the levels of GSH, TBARS and activities of superoxide dismutase (SOD), glutathione-*S*-transferase (GST) and catalase (CAT). *Results*: Restraint stress induced a decrease in the level of GSH and the activities of SOD, GST and catalase, while the levels of TBARS were found elevated. Both pre-stress and post-stress vitamin treatments (either alone or combined) resulted in alteration of these parameters towards their controls values with a relative dominance by latter. Vitamin E was found most effective in restoring inherent antioxidant system, no additive effect was observed in combined vitamin treatment as expected. *Conclusion*: Immobilization of rats generated oxidative stress in rat brain, by decreasing the activities of SOD, GST, catalase and glutathione levels, while increasing the lipid peroxidation. Post stress vitamin E treatment was found most effective than vitamins A and C in enhancing the levels of glutathione and activities of SOD, GST and catalase and decreasing lipid peroxidation. Thus vitamin E can be given as a nutritional supplement for scavenging free radical generated in the brain tissues in order to reduce oxidative stress.

Keywords: Immobilization stress; GST; SOD; Catalase; GSH and MDA; Vitamins A, E, C

1. Introduction

Several reports have indicated that stress affects synaptic plasticity, dendritic morphology and neurogenesis in animals [1] and induces both clinical and anatomical features of neurotoxic damage in humans (i.e. posttraumatic stress disorders) [2]. The precise mechanism by which stress induces brain damage is still a matter of debate. Both constitutive formation of NO and inducible expression of iNO synthase have been found to occur in the brain during chronic stress [3]. Reactive oxygen species are closely involved in several diseases of the nervous system including

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Parkinson's disease, Schizophrenia, and Alzheimer's disease [4].

Immobilization stress is an easy and convenient method of inducing both psychological (escape reaction) and physical stress (muscle work) resulting in restricted mobility and aggression [5,6]. Recently various stresses have been associated with enhanced free radical generation causing oxidative damage [7]. Of all the organs in the body, the CNS takes more than its share of oxidative abuse [8,9]. The main factors that contribute to the vulnerability of brain to oxidative damage include high content of polyunsaturated fatty acids in the membranes and low levels of enzymatic and nonenzymatic antioxidants [10]. Moreover, stress has been shown to cause a decrease in the level of glutathione (GSH) and vitamin C, which protect the tissues from oxidative damage [11]. Apart from polyphenols, especially tocopherols, carotenoids and ascorbic acid have been associated with antioxidative properties [12].

In the present study, the effect of oxidative stress generated by restraint stress on brain tissues was evaluated in terms of measurement of free radical scavenging enzymes like superoxide dismutase (SOD), catalase (CAT), glutathione-*S*-transferase (GST), glutathione and lipid peroxidation (thiobarbituric acid reactive substances) The antioxidative potential of vitamins A, E, C alone and in combination (vitamins E and C) was also studied on restraint stress induced oxidant/pro-oxidant status of the rat brain.

2. Materials and methods

Adult male Wistar rats weighing 180–200 g were housed in group cages for the study. Purina diets and tap water were supplied to them ad libitum. All the experimental protocols adhered to the guidelines of the animal welfare committee of the university. Prior to the commencement and throughout the experiment the rats were housed at 24 ± 3 °C room temperature and 12 h light/dark cycles.

Vitamin A (gelatin capsule as palmitate 25,000 IU equivalent to retinol 7.5 mg) and vitamin E (α-tocopheryl acetate 544 IU as 400 mg gelatinous capsule) were from Merck, Goa, India. All are analytical grade reagents and chemicals.

Rats were exposed to stress between 9 AM and 3 PM in the animal house. Immobilization stress was accomplished by placing individual animals in wire mesh cages of their size attached to a wooden board. The rats were deprived of food and water during stress exposure [6]. The animals were subjected to 6 h stress, and then sacrificed after 30 min by giving pentobarbital (i.p. 50 mg/kg of body weight). Control animals were handled at the same time as the stressed and were placed in individual cages during the corresponding time.

The animals were divided into two broad groups for the stress and vitamins A, E, C and E and C treatment studies. One group received vitamin A (50,000 IU), E (19.40 IU), C individually and combined vitamins (E and C) (15 mg/kg of body weight) dissolved in olive oil 30 min prior to 6 h stress session (pre-vitamin stress treated), while the other groups received similar doses of vitamins 30 min after the stress session (post-vitamin treated). The controls for this group received olive oil alone prior to and after stress session to rule out the effects of olive oil (vehicle alone). The rats were sacrificed 30 min after the termination of the experiment by giving sodium pentobarbital as discussed above. Brains from all the animals were taken out quickly, washed with chilled n-saline and homogenized or sonicated immediately to give 10–20% homogenate, respectively, in tris–HCl buffer (0.2 mol/l, pH 7.0)/phosphate buffer (0.1 mol/l, pH 7.0) for biochemical estimations. The homogenate was centrifuged at 8000 × *g* for 5 min at 4 °C to separate the nuclear debris and supernatant was collected.

The brain homogenate was subjected for the assay of free radical metabolizing enzymes catalase [13], SOD [14], glutathione-*S*-transferase [15], reduced glutathione [16] TBARS [17] at 37 °C using a spectrophotometer by standardized procedures. Protein was measured by Lowry et al. The GSH content of the brain was expressed as per gram of tissue.

3. Statistical analysis

One way ANOVA test at $p = 0.05$ was used on the data obtained by the repeated investigations. Paired *t*-test were also performed at $p < 0.05$ to decide whether the results are significantly changed or not (followed

Table 1
Effect of vitamins A, E and C treatments individually and in combination (vitamins E and C) on the brain tissue activities of SOD, GST, catalase and the levels of lipid peroxidation and glutathione in restraint stress treated rats (Mean ± S.E.M.)

		SOD (units/mg of protein)	Catalase (units/mg of protein)	GST (nmol/mg of protein)	MDA (nmol/mg of protein)	GSH (μmol/g tissue)
Control rats (10)		3.35 ± 0.04	7.38 ± 0.35	141.23 ± 9.03	7.93 ± 0.60	11.32 ± 0.985
Stressed rats (10)		$1.98^{cc'}$ ± 0.04	$5.39^{cc'}$ ± 0.63	$103.53^{cc'}$ ± 0.017	$11.37^{cc'}$ ± 0.62	$5.84^{cc'}$ ± 0.678
Pre-vitamin stress treated	Vit A (10)	2.10^{ab} ± 0.13	5.50^{bc} ± 0.65	112.65^{ch} ± 3.44	11.01^{cb} ± 0.34	$6.13^{a'a}$ ± 0.496
	Vit E (10)	2.30^{bc} ± 0.13	5.90^{ch} ± 0.32	119.39^{cc} ± 1.07	10.35^{bc} ± 0.21	7.01^{bc} ± 0.671
	Vit C (10)	$2.20^{a'b}$ ± 0.13	5.85^{ba} ± 9.5	118.89^{ac} ± 1.14	10.64^{ab} ± 0.88	6.64^{ab} ± 1.09
	Vit (E and C) (10)	2.33^{ac} ± 0.12	$5.96^{a'b}$ ± 0.56	120.58^{bc} ± 1.74	$10.32^{a'b}$ ± 0.53	7.10^{bc} ± 0.865
Post-vitamin stress treated	Vit A (10)	2.21^{ab} ± 0.13	5.7^{ab} ± 0.94	118.696^{ac} ± 1.21	10.34^{ba} ± 0.39	$6.89^{a'b}$ ± 1.035
	Vit E (10)	$2.28^{a'b}$ ± 0.13	6.3^{ac} ± 0.89	129.96^{ab} ± 2.11	9.63^{ab} ± 0.26	9.93^{ac} ± 1.351
	Vit C (10)	2.25^{ab} ± 0.14	6.1^{bc} ± 0.72	$124.34^{a'b}$ ± 1.88	10.13^{bc} ± 0.60	7.86^{ab} ± 0.489
	Vit (E and C) (10)	$2.30^{a'b}$ ± 0.17	$6.31^{c'b}$ ± 0.79	130.01^{cb} ± 3.22	$10.10^{c'b}$ ± 0.75	10.12^{ac} ± 1.23

Number of experimental rats are indicated in the parenthesis. a,a′, $p<0.05$; b,b′, $p<0.01$; c,c′, $p<0.001$ where a, b, c as compared to nonstressed control rats and a′, b′, c′ to stress treated rats.

by pairwise comparison Tukey's honestly significant post hoc analysis). Similar statistical analyses were also performed for the enzyme activities from pre- and post-vitamin stress treatments with respect to stress alone or nonstressed controls. The results are summarized in Table 1.

4. Results

The present study revealed that 6 h of restraint stress caused a significant decrease in the brain activities of SOD ($F_{1,9}=9.943$, $p<0.01$), GST ($F_{1,9}=13.631$, $p<0.01$), catalase ($F_{1,9}=15.036$, $p=0.001$) and the levels of glutathione ($F_{1,9}=19.036$, $p<0.05$) with a significantly increased levels of TBARS ($F_{1,9}=15.432$, $p<0.005$) in comparison to nonstressed control rats. A single dose of olive oil (1 ml/kg body weight) did not cause significant change in these biochemical parameters (results not shown). Oral administration of vitamin A, and C alone/or both before (pre-vitamin stress treatment) and after (post-vitamin stress treatment) immobilization stress treatment resulted in a less significant alteration of these parameters as compared to stress treated rats.

Vitamin E treatment alone, or both prior to and after restraint stress resulted in a significant alteration in the brain levels of these enzymes towards their control values. However, the post-stress oral administration of vitamin E (15 mg/kg body weight) was found more effective in restricting stress induced decrease of SOD ($F_{1,9}=6.422$, $p<0.05$), GST ($F_{1,9}=8.952$, $p<0.05$), CAT ($F_{1,9}=11.352$, $p=0.001$), glutathione ($F_{1,9}=13.234$, $p<0.05$) and increase in the level of TBARS ($F_{1,9}=6.451$, $P<0.05$) as compared to stress alone or other vitamin treatments. The combined vitamins (E and C) treatment did not result in any additive antioxidant effect as expected, rather an effect similar to vitamin E alone was observed.

5. Discussion

All cells in the body are exposed chronically to oxidants from both endogenous and exogenous sources but are also equipped with an antioxidant system. Reactive oxygen and nitrogen species, if unchecked, can contribute to chronic disease development by oxidatively modifying lipids, nucleic acids and proteins [18]. Nutrients, both water-soluble and lipid soluble comprise an important aspect of the antioxidant defense system. Of all the organs, the brain is thought to be vulnerable to oxidative damage due to its high oxygen consumption, presence of high levels of polyunsaturated fatty acids (PUFA) and nondegenerative nature of neurons [19], which may lead to various neurodegenerative diseases [19].

Restraint/immobilization stress is a well-known method for the production of chronic physical and

emotional stress [6]. Six hours of immobilization stress resulted in the generation of oxidative stress/reactive oxygen species (ROS) in the brain of rats by decreasing the levels of GSH and the activities of enzymes SOD, GST and catalase, while enhancing the level of thiobarbituric acid reactive substances (TBARS), which is an indicative of lipid peroxidation. The ROS may propagate the initial attack on lipid rich membranes of the brain to cause lipid peroxidation (LPO) [20]. The enhanced LPO may also be due to marked depletion of GSH content of brain, which acts as one of the guarding factors against oxidative stress [21]. The glutathione depletion might be the result of decreased activities of GST, SOD and catalase, the free radical scavenging enzymes. Restraint stress has been shown to bring about antioxidant defense changes in the plasma of rats [22]. The already known poor catalase and moderate glutathione peroxidase and SOD activities of brain [23] are found further reduced by restraint stress. GST also possesses peroxidase activity and can directly attack the peroxides that may be generated via oxidative reduction recycling, resulting in decreased toxicity [24]. But the decreased GST activity as observed in the present study might have further contributed to the enhanced lipid peroxidation as depicted by increased TBARS levels. Thus, immobilization stress is found capable of generating oxidative stress in rat brain.

Both natural nonenzymatic ex vivo (vitamins A, E and C) and in vivo SOD, GST, catalase and glutathione [14–16] antioxidant defense mechanism exist. However, the antioxidant mechanism fails either due to overproduction of free radicals or decreased activities of scavenging enzymes, or both causing lipid peroxidation. Since lipid peroxidation is a self-propagating chain reaction the initial oxidation of only a few lipid molecules can result in significant tissue damage and disease, especially so in PUFA rich brain tissues. Glutamate, dopamine, iron-catecholamine complexes and reactive oxygen species are involved in several diseases of the nervous system including Parkinson's disease, Schizophrenia and Alzheimer's disease [4]. In all these oxidative stress and a failure of antioxidant defense are found involved.

A potential role for the antioxidant micronutrients vitamins A, E and C in modulating oxidative stress generated by restrained stress may determine their clinical usefulness as supplemental nutritional therapeutic agent in disorders affecting the brain free radical metabolism. The treatment of rats both prior to or after stress with these vitamins alone or in combination (vitamins E and C) resulted in an increase in the activities of SOD, GST, catalase and levels of reduced glutathione with a decrease in lipid peroxidation. Post-vitamin treatments were found more effective in combating stress induced pro-oxidant changes than pre-vitamin treatments. Vitamins A, E and C are reported to act as an effective antioxidant of major importance for protection against diseases and degenerative processes caused by oxidative stress [25,26]. The present study has revealed a predominant effect of vitamin E in restoring or restricting the restraint stress induced oxidative derangements in rat brain.

The antioxidant potential of vitamins (E and C) together though was found effective but not additive as expected; rather a predominant effect similar to vitamin E alone was observed. Thus, vitamin E appears to be the first line of defense against peroxidation of PUFA present in cellular and subcellular membrane phospholipids.

The acetylcholinesterase inhibitors are reported to give only a temporary relief in Alzheimer's disease [27], still definitive clinical trials are required to reduce both neurotoxic, neuroinflammation and oxidative stress. The results of the present study suggest that the dietary supplementation of vitamin E may be effective in combating the neurodegenerative diseases. With initial large doses to build up tissue levels followed by the lessening of supplementation may be effective in both prevention and therapeutic efficacy of these vitamins. Similar to our findings, a high dose of vitamin E dietary supplementation or parenteral vitamin E administration (e.g. vitamin E succinate) is proposed to serve as a successful therapeutic strategy for the prevention or treatment of Parkinson's disease [28].

In addition, studies have suggested that supplementation with at least 100 mg/day of vitamin E may decrease the risk of heart disease [29]. This is well above the current RDA and is far greater than can be obtained from even a very well balanced diet. These findings have rekindled the debate as to whether dietary recommendations should consider optimal levels of these vitamins rather than the levels needed to prevent lipid peroxidation. As a fat-soluble vitamin,

E has the potential for toxicity. However, it does appear to be the least toxic of the fat-soluble vitamins. No instances of toxicity have been reported at doses of ≤ 1600 mg/day [30].

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